PRODUCTION OF ANTIBODIES FOR THE MEASUREMENT OF HUMAN SERUM LIPOPROTEINS

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To My Father

We accept this thesis as conforming to the

Required standard for the degree of

Master of Biochemistry

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Abstract

Coronary heart disease (CHD) is a chief cause of death in western countries. It has been suggested that apolipoprotein A-I (apoA-I) and apolipoprotein B100 (apoB100), the principal protein component of high density lipoprotein (HDL) and low density lipoprotein (LDL), respectively, are the most significant parameters for assessment of CHD risk. Our laboratory has tried to set up an immunodiagnostic assay for measurement of serum apoA-I and apoB100.

Human apoA-I and apoB100 were purified from serum. Rabbits were immunized with these apolipoproteins for polyclonal antibody production. Hybridoma cell lines were produced which secret anti-apoA-I and anti-apoB100 monoclonal antibodies. These monoclonal antibodies are only specific to their target lipoproteins and they bind identically to purified apolipoproteins and native lipoproteins.

Using the monoclonal and polyclonal antibodies, a sandwich ELISA was developed for the measurement of apoA-I. In this assay, microtiter plates were pre-coated with protein A. Polyclonal antibodies were immobilized onto the solid phase through the binding with protein A. Monoclonal antibodies were conjugated to alkaline phosphatase as detecting antibody. The assay has a working range of 0.2 to 1.2 μg/ml of apoA-I and the intra- and inter-assay variations are 5.96 and 7.83%, respectively.

Abbreviation

2-MEA	2-Mercaptoethylamine
ApoA-I	Apolipoprotein A-I
ApoB100	Apolipoprotein B100
CHD	Coronary Heart Disease
DMSO	Dimethyl Sulfoxide
EDTA	Ethylene DiamineTetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
cFA	Complete Freund's Adjuvant
iFA	Incomplete Freund's Adjuvant
FCS	Fetal Calf Serum
HAT	Hypoxanthine Aminopterin Thymidine
HDL	High Density Lipoprotein
IDL	Intermediate Density Lipoprotein
IgG	Immunoglobulin G
LCAT	Lecithin Cholesterol Acyltransferase Transferase
LDL	Low Density Lipoprotein
LPDS	Lipoprotein Depleted Serum
PBS	Phosphate Buffer Saline
PBSMT	Phosphate Buffer Saline (with 3% dry Milk, 0.1% Tween20)
PBST	Phosphate Buffer Saline (with 0.1% Tween20)
PEG	Polyethylene Glycol
PMSF	Phenylmethyl Sulfonyl Flouride
pNpp	p-Nitrophenylphosphate
RCT	Reverse Cholesterol Transport
SDS-PAGE	Sodium Dodecylsulphate-Polyacrylamide gel electrophoresis
SMCC	N-Succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate
TFA	Trifluoroacetic Acid

Contents

Chapter 1

Introduction to Lipoprotein and Apolipoprotein

1.1 Lipoprotein structure and classification

Lipoproteins are water-soluble macromolecular complexes of lipids (triglycerides, cholesterol and phospholipids) and specific proteins, referred to as apolipoproteins. A major function of the lipoproteins is to transport lipids through the vascular and extravascular body fluids. Their general structure is globular. Within the core of the lipoprotein particle are the more hydrophobic lipids, viz, the esterified cholesterol and the triglycerides. On the outer layer are the more polar lipids, such as phospholipids, free cholesterol and the apolipoproteins; with their charged groups pointing out towards the water molecules.

Human plasma lipoproteins are commonly divided into five major classes based on their densities (Figure 1.1). They are chylomicrons, very low density lipoproteins (VLDL, d<1.006g/ml), intermediate density lipoproteins (IDL, d=1.006-1.019g/ml), low density lipoproteins (LDL, d=1.019-1.063g/ml) and high density lipoproteins (HDL, d=1.063-1.21g/ml).

1.2 Apolipoprotein A-I and B100

1.2.1 Apolipoprotein A-I

Apolipoprotein A-I (apoA-I) is the major protein component of HDL. Its primary structure was first reported by Brewer et al. (Brewer et al. 1978). It is a single polypeptide chain of 243 amino acid residues with a molecular weight of 28 kDa. It contains approximately 55% alpha helix, 8% pleated sheet, and 37% random coil. ApoA-I is synthesized as a preproapolipoprotein with 24 amino acids attached

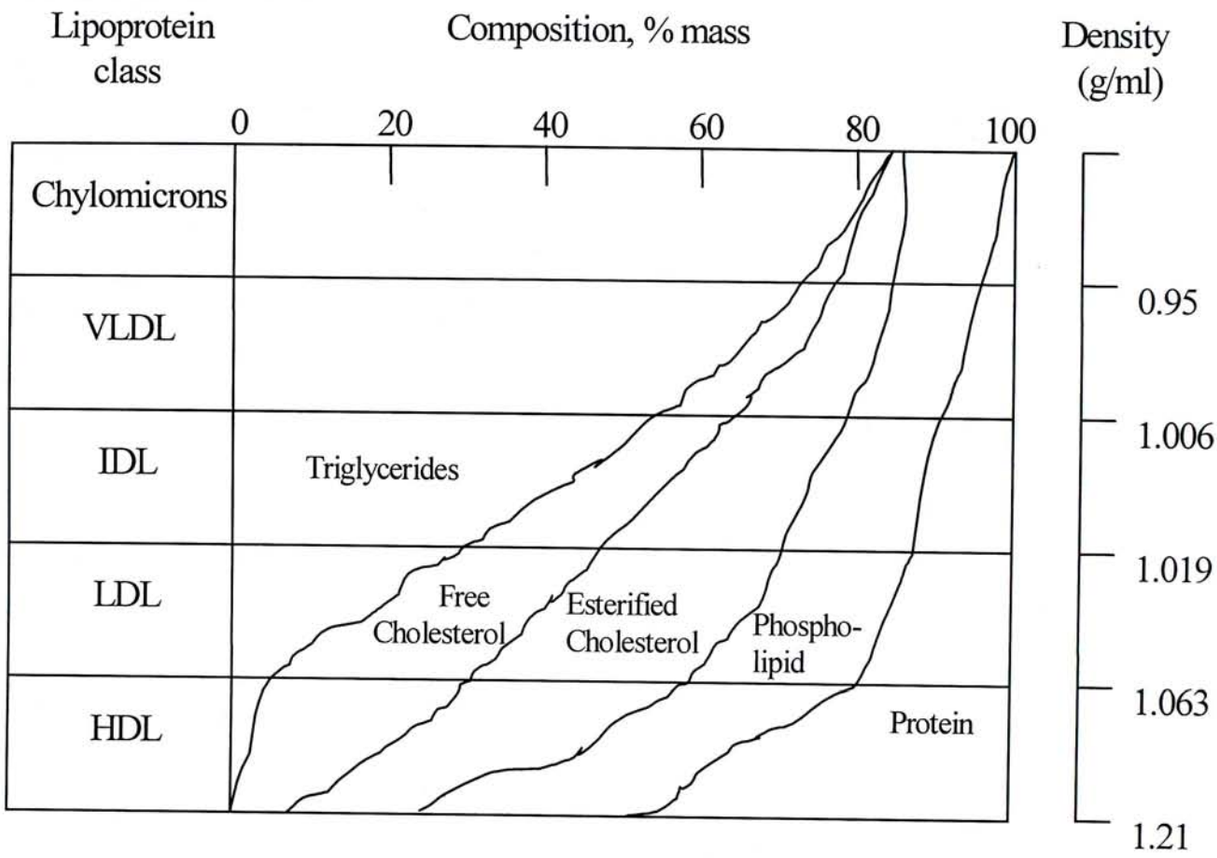


Figure 1.1 The classification, composition, and density of the lipoproteins

to the N-terminus of the mature protein (Duverger et al. 1993; Law and Brewer 1984). PreproapoA-I undergoes co-translational cleavage of the N-terminal 18 amino acids to produce proapoA-I. ProapoA-I, containing the propeptide sequence Arg-His-Phe-Trp-Gln-Gln, accounts for approximately 4% of the total fasting plasma apoA-I. There are other possible post-translational covalent modification reported in the literature. These include phosphorylation and myristoylation (Law and Brewer 1984). Polymorphism of apoA-I, demonstrated by isoelectric heterogeneity, is in most cases thought to be the result of post-translational modification. ApoA-I has been separated into four polymorphic forms by isoelectric focusing and into six forms by two-dimensional electrophoresis (Nestruck et al. 1980; Zannis et al. 1980).

1.2.2 Apolipoprotein B100

ApoB100 is a major protein component of VLDL, IDL, LDL, and a unique lipoprotein, lipoprotein (a). ApoB100 is one of the largest proteins known, containing 4536 amino acid residues with a molecular weight of 550 kDa (Chen et al. 1986; Knott et al. 1986; Law et al. 1986). ApoB100 has 19 potential N-linked glycosylation sites, of which only 16 are actually glycosylated (Yang et al. 1989). ApoB100 contains 8-10% carbohydrate in the form of galactose, mannose, N-acetylglucosamine, and sialic acid residues (Swaminathan and Aladjem 1976). Analysis of secondary structure indicates that apoB100 contains 43% of alpha helix, 21% β-sheet, 20% random structures, and 16% β-turns (Garnier et al. 1978). There are twenty five cysteine residues in apoB100 which are quite asymmetrically distributed and are located mainly in the N-terminal region. Sixteen of the cysteine residues are involved in disulfide linkage (Yang et al. 1990). Immunoelectron

microscopic analysis of apoB100 on LDL suggests that the protein is extended and spans at least a hemisphere of the lipoprotein particle surface (Chatterton et al. 1991).

1.2.3 Biological functions of apolipoprotein

It is now established that the apolipoproteins of the various lipoproteins regulate lipoprotein metabolism and determine their unique roles in lipid metabolism (Table 1.1). Several major functions have thus far been ascribed to specific apolipoproteins. One well-established function is their involvement in the transport and redistribution of lipids among different tissues. The delivery of lipids to specific cells involves the recognition of specific apolipoproteins by cell surface lipoprotein receptors. ApoB100 has been shown to mediate the interaction of lipoproteins with LDL receptors of the liver and extrahepatic tissues (Chan 1992; Mahley 1988). A second function of specific apolipoproteins involves their role as cofactors for enzymes of lipid metabolism. The LCAT (lecithin:cholesterol acyltransferase) reaction is activated by apoAI (Ikeda et al. 1994). This cofactor appears to activate LCAT by providing a suitable lipid or liposome interface upon which the enzyme can work. An additional function for apolipoproteins involves their role in the maintenance of the structure of the lipoproteins. Various apolipoproteins, e.g., apoB100 and apoA-I, appear to stabilize the micellar structure of the lipoproteins and function, in association with phospholipids on the surface of the particles, to provide a hydrophilic surface.

1.3 Evidence linking apoA-I and B100 with atherosclerosis

The strong association between risk of coronary heart disease (CHD) and apolipoproteins has been well documented in several large epidemiological studies

Table 1.1 Apolipoproteins: their properties and functions

Apolipoprotein	Molecular mass	Plasma concentration, mg per 100 ml	Function
AI	28 016	80-160	Activation of LCAT; Detergent properties
AII	17 414	20-55	? Activation of hepatic lipase
B48	264 000	0-2	Secretion of chylomicrons
B100	550 000	60-160	Secretion of VLDL; Structural protein of LDL; Receptor-mediated LDL catabolism
CI	6 600	3-11	?
CII	8 850	1-7	Activation of lipoprotein lipase
CIII	8 800	3-23	? Inhibition of hepatic uptake of chylomicrons and VLDL
E	34 100	2-6	Hepatic clearance of chylomicron remnants and IDL
(a)	300 000-700 000	1-100	? Inhibitor of fibrinolysis

(Stampfer et al. 1991; Levinson and Wagner 1992; Sigurdsson et al. 1992; Coleman et al. 1992). All of these studies drew the same conclusion; i.e., ApoA-I is significantly lower and apoB100 significantly higher in myocardial infarction survivors.

Transgenic mice expressing human apoA-I and apoB100 have previously been created (Rubin et al. 1991a; Callow et al. 1994). The C57BL/6 mice expressing the human apoA-I transgene had higher plasma concentrations of human apoA-I and HDL than non-transgenic mice and were resistant to diet-induced atherogenesis (Rubin et al. 1991b). The presence of the high expressing human apoB100 transgene was associated with a 2.5-fold increase in LDL cholesterol and a 15-fold increase in proximal lesions of the coronary arteries compared with non-transgenic mice (Callow et al. 1995).

1.4 The roles of apoA-I and B100 in the development of atherosclerosis

In seeking a mechanism for the anti-atherogenic effect of HDL, most attention has been directed at the role of HDL in reverse cholesterol transport (RCT) (Oram et al. 1987; Hara and Yokoyama 1992; Fruchart et al. 1994). The interaction of HDL with its receptor appears to facilitate the transport of cholesterol from cells to HDL particles. Ligand blotting study has provided evidence that this receptor is a 110 kDa membrane protein (Schmitz et al. 1985). Binding of apoA-I to this receptor can stimulate the production of diacylglycerol which can activate protein kinase C and promote the translocation of cholesterol from intracellular compartments to the apoA-I containing lipoprotein (Figure 1.2) (Theret et al. 1990). The receptor-bound HDL particles are then released back into the extracellular fluid. This reversible

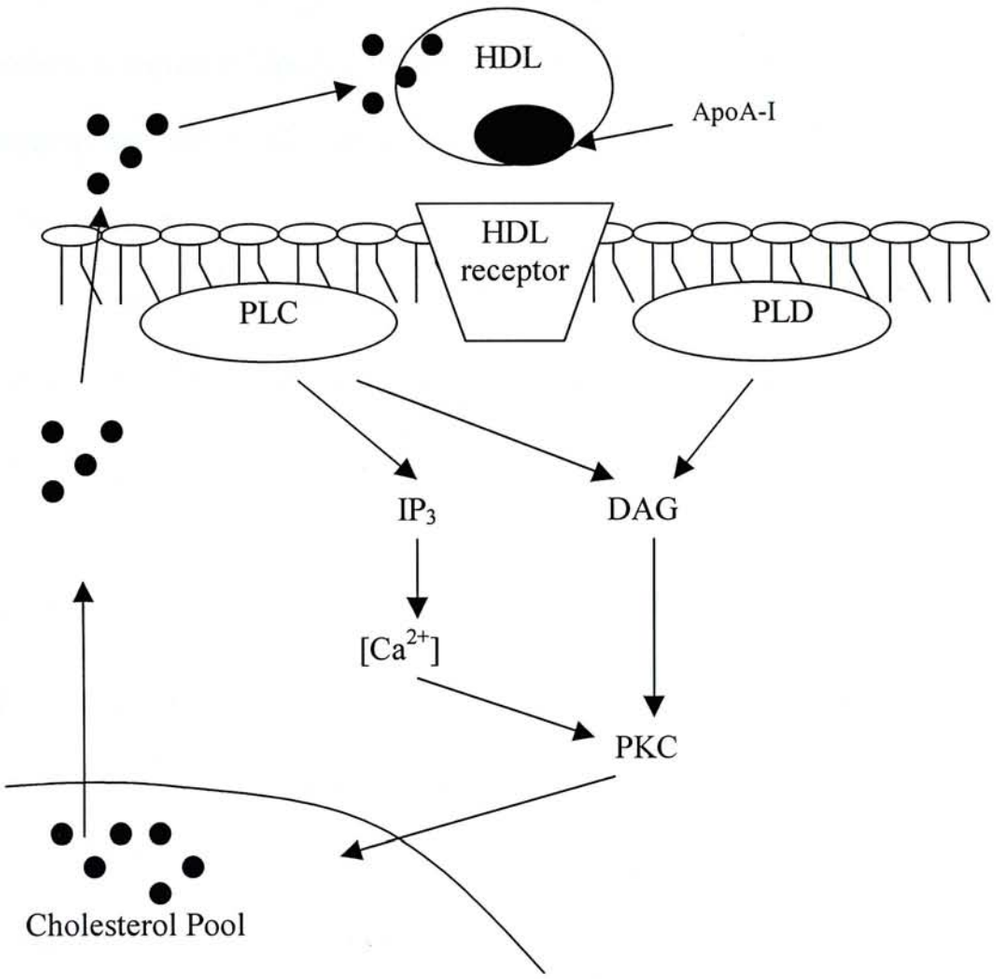


Figure 1.2 ApoA-I mediated Reverse cholesterol transport. ApoA-I in the HDL is proposed to bind to HDL receptor, and triggers a signaling pathway to translocate intracellular cholesterol to cell membrane. HDL, itself acts as cholesterol acceptor and transport these excess cholesterol to liver.
PLC, phospholipase C; PLD, phospholipase D; IP_3, inositol trisphosphate; PKC, protein kinase C

association allows HDL to sequester cholesterol and remove it from the cell. In a recent study, synthetic peptides can mimic apoA-I in both binding to cell-surface receptors and clearing cholesterol from cells (Mendez et al. 1994). Using monoclonal antibodies, a region of apoA-I within or adjacent to residues 140-150 is found to be responsible for the ability of apoA-I to promote intracellular cholesterol efflux (Sviridov et al. 1996).

The common initiating step of the LDL oxidation is the peroxidation of the polyunsaturated fatty acid within the LDL molecule (Steinberg et al. 1989), probably as a result of the production of oxygen free radicals. Lipid peroxides formed in this way spontaneously decompose to short-chain aldehydes which can attach covalently to apoB100 amino-acid residues (Fong et al. 1987), particularly the lysine amino groups (Steinbrecher 1987). Besides the LDL receptor, macrophage has another receptor, the scavenger receptor, which is responsible for the accumulation of oxidized LDL and the formation of foam cell (Figure 1.3). The chemical change to apoB100, and not the lipid moiety of the particle, is the crucial component recognized by the scavenger receptor (Parthasarathy et al. 1987). This scavenger pathway – unlike the normal LDL receptor – is not down regulated when the cell has sufficient lipid for its own maintenance (Nagelkerke et al. 1983). Therefore, macrophages can become literally bloated with lipid droplets if there is enough modified LDL in the nearby extra-cellular fluid.

1.5 Measurement of human serum lipoproteins as an assessment of risk for coronary heart disease (CHD)

In the last several decades, it has been customary to utilize the measurement of LDL and HDL cholesterol in profiling the CHD risk of an individual (Kannel et al.

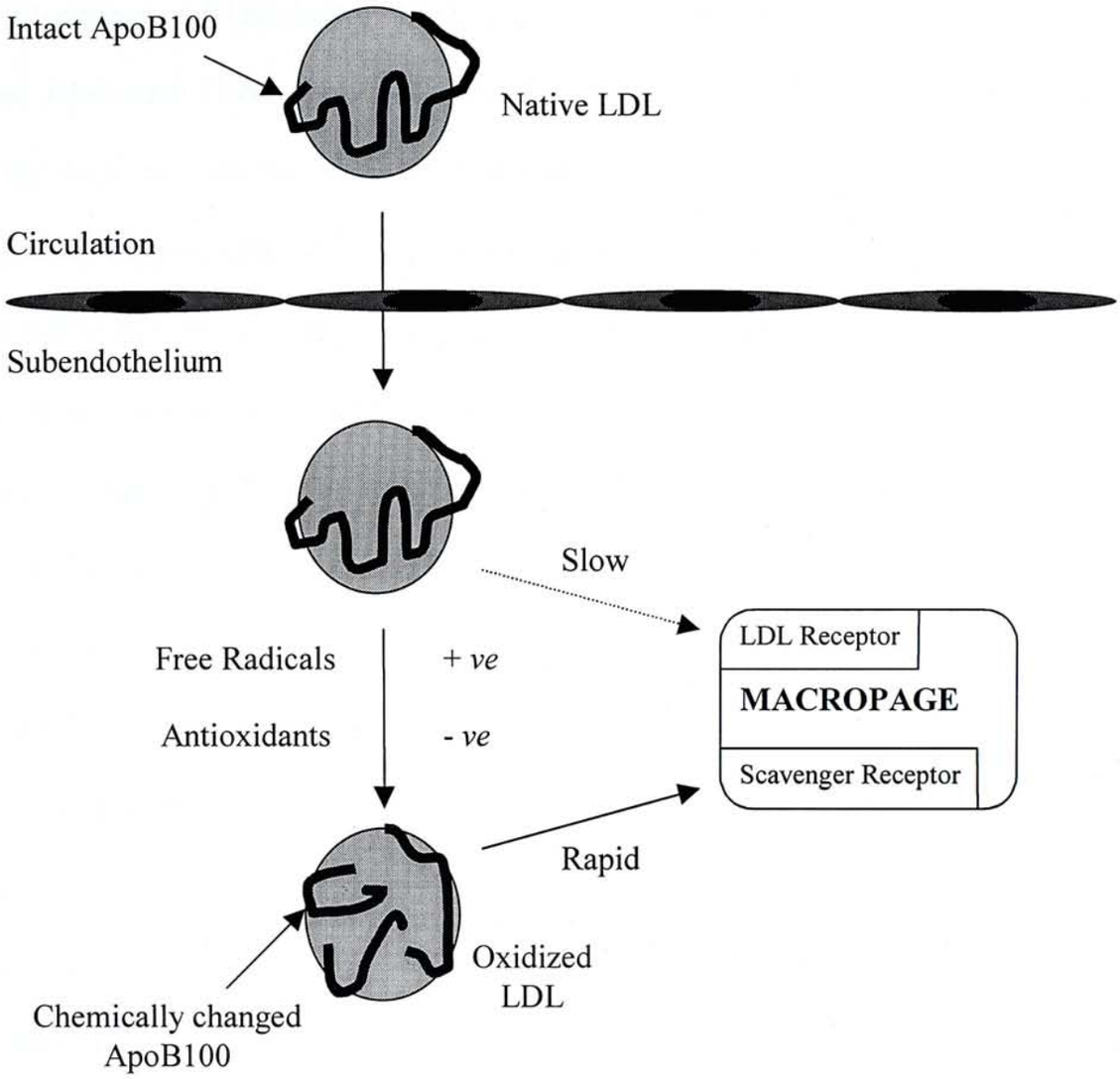


Figure 1.3 The 'oxidative modification theory' of atherosclerosis. Native cholesterol-rich low density lipoprotein (LDL) particles intermittenly traverse the endothelium to enter the vascular wall. Once in the subendothelium, they may become oxidized and taken up locally by the scavenger receptor of macrophages. This process leads eventually to cholesterol loading and formation of foam cells characteristic of early atherosclerotic lesions.

1971). Of the recent biochemical markers added to the constellation of atherogenic index, the apolipoproteins, especially apoA-I and apoB100, appear to be the most straight forward indicators for use in the clinical laboratory (Srinivasan and Berenson 1995; Bhatnagar and Durrington 1991; Laker and Evans 1996). In the editorial of *Clinical Chemistry* (1996, issue 42), Dr. Sniderman concluded, quoted, 'After so much emphasis on cholesterol as the hallmark for risk assessment, is it possible to change to apolipoproteins without confusion and loss of credibility? If change is in the patient's interest, if change leads to more precise diagnosis and treatment, if change makes our practice simpler and in the end less expensive, should we not welcome change rather than resist it?' (Sniderman and Cianflone 1996). The reference limits for apoA-I and apoB100 as proposed by the Framingham offspring study are both at 1.2g/L (Contois et al. 1996a; Contois et al. 1996b). Subjects with serum apoA-I level lower than 1.2g/L or apoB100 higher than 1.2g/L are more likely to have CHD than subjects with apoA-I higher than 1.6g/L or apoB100 lower than 1.0g/L.

1.6 Aims of this study

When immunoassay was first introduced for measuring apolipoproteins in serum (Schonfeld and Pfleger 1974), antibodies specific to apolipoprotein A-I were polyclonal since they were raised in animals. The animal, usually a rabbit or a goat was immunized and bled. The resultant antiserum was used as the reagent. The immunoglobulin G made from these antisera carried a mixture of several hundred different types of antibodies. Each of these antibodies has its own characteristics and hence, the property of polyclonal antibody is heterogeneous. Consequently, standardization of the immunoassays that based on antisera is difficult. To overcome

the above problems, monoclonal antibody could be a useful alternative. With its pre-defined specificity, cross-reactions due to mixture of antibodies against irrelevant antigens could be eliminated.

In this study, both polyclonal and monoclonal antibodies against apolipoprotein A-I and B100 will be produced. They will be used to set up a direct ELISA for the quantification of apoA-I and B in human serum. The antibodies, before being employed, will be characterized mainly in terms of specificity. A major problem in solid phase immunoassays is the denaturation of the protein ligand bound onto a plastic surface. To overcome this problem, an alternative ELISA method, protein A-coated antibody capture ELISA has also been developed.

Chapter 2

Purification of Apolipoprotein A-I and B100 and Production of Polyclonal Antibodies

2.1 Introduction

2.1.1 Purification of apolipoprotein A-I and B from human serum

Human serum contains over 100 proteins ranging in concentration from 40 mg/ml (albumin) to almost undetectably low levels. Table 2.1 shows the major human serum proteins, their normal concentrations and biological functions.

ApoA-I is the major protein of HDL, comprising about 70% of the total HDL proteins. Isolation of apoA-I generally includes ultracentrifugation to isolate HDL, delipidation with organic solvent to obtain apoHDL proteins free of lipid, and various chromatographic separation of apoA-I from other HDL peptides such as gel filtration, ion exchange (Beg et al. 1989; Law et al. 1983) or preparative reversed phase HPLC (Hughes et al. 1988).

The method for apoB100 isolation is quite similar to apoA-I. ApoB100 purification is more difficult than apoA-I due to its extreme size and lability. Moreover, apoB100 is highly hydrophobic and insoluble in aqueous solutions. Attempts to solubilize apoB100 in aqueous buffers by using chemical modification- i.e., succinylation or maleylation- have resulted in a low yield of soluble protein (Gotto et al. 1968; Scanu et al. 1968). Another way to solubilize apoB100 is the addition of detergents or guanidine-HCl to stabilize the apolipoprotein in solution (Socorro and Camejo 1979; Steele and Reynolds 1979).

Table 2.1 Major human serum proteins

Protein	Molecular Mass	Normal concentration, mg per 100 ml	Biological functions
Albumin	66 000	3500-5500	Osmotic function; protein reverse, transport of ions, pigments, etc.
Immunoglobulin G	150 000	800-1700	Major serum and tissue antibody; When aggregated activates both pathways of complement
Immunoglobulin M	970 000	50-320	First antibody to appear after antigenic stimulation; When bound to antigen activates classical pathway of complement
Immunoglobulin A	160 000	100-500	Antibodies with restricted specificity; Poor activator of alternative pathway of complement
Haptoglobin	86 000	400	Hemoglobin binding
Fibrinogen	341 000	200-450	Precursor of fibrin, major protein of clots
Transferrin	80 000	200-400	Iron binding and transport; Unsaturated transferrin is bacteriostatic
Apolipoprotein A-I	28 020	115-210	Main protein component of HDL; Cofactor of LCAT; Stabilizes prostacyclin, reduces platelet activation
Apolipoprotein B100	550 000	65-155	Main protein component of LDL and VLDL; Regulates lipoprotein metabolism
α1-Antiproteinase inhibitor	54 000	200-400	Inhibitor of neutrophil elastase also trysin, chymotrypsin
α2-Macroglobulin	820 000	150-350	Inhibits most endoproteinases

2.1.2 Immunization for polyclonal antibodies production

There is no single method for animal immunization that guarantees an ideal product. Nevertheless, certain principles can be adopted which form the ground rules for an immunization protocol.

For practical reasons, rabbits is a good choice for the routine production of polyclonal antisera. However, most laboratory rabbits are outbred and, hence, they respond individually, so more than one animal should be used for immunization.

The immunogenicity of an antigen can be enhanced by the addition of adjuvant. Most adjuvants are of two main types: colloidal (e.g. aluminium hydroxide), or oils that can be used to form an emulsion with an aqueous antigen preparation (e.g. Freund's complete and incomplete adjuvants). These are mixed thoroughly with the immunogen prior to immunization. This is important, as it has been calculated that only 0.1% of the antigen administered in a single dose actually reaches the immune system in the absence of adjuvant. Some adjuvants contain additional components that enhance immune response. For example, complete Freund's adjuvant (cFA) contains heat-killed mycobacteria in addition to oil and an emulsifying agent. Complete Freund's adjuvant should be used for the first immunization but should be replaced by incomplete Freund's adjuvant (iFA, oil and emulsifying agent only) for all subsequent immunization to avoid excessive inflammation.

For a soluble protein immunogen mixed with Freund's adjuvant, a dose of 0.5-1.0 mg/animal is recommended for rabbit immunization (Harlow and Land 1988). The route of injection should also be considered and guided by the injection volume, components of the immunogen and the desired rate of release. For the rabbit, large volume injections are normally given at multiple subcutaneous sites on the back. If

slow release of the immunogen is desired then the injection should be intramuscular. After initial (or priming) injection, the majority of antibodies produced belong to the class M immunoglobulins (IgM). This is known as the primary response. The initial priming of the host animals will establish the immuno-memory for this particular antigen. If the antigen is reintroduced later, the antibody response is faster, stronger, persists for a longer period and is qualitatively different. This is known as the secondary response. The most important observation in this secondary response is the shift of antibody class from IgM to immunoglobulin G (IgG). The response of the third and subsequent injection broadly resembles that of the secondary response (Figure 2.1). The length of time between immunization can influence the affinity of the antibodies. After immunization and subsequent initial antibody production, antigen levels will become progressively reduced. When a low concentration is reached, only high affinity B cells will be activated and clonally expanded. The longer the interval between immunizations, the lower the residual antigen and the greater the preferential selection for high-affinity B cells. The interval between the initial and other boosters is recommended to be between 3-6 weeks (Harlow and Land 1988).

2.1.3 Antibody purification

If the antibody is to be used without a label being attached to it, partial purification of it may be sufficient. If it is to be labeled, then it should be more than 80% pure or the labeling process will be inefficient. If the antibody is to be used for *in vivo* application, then it must be free of any toxins (pyrogens). The strategy for antibody purification also depends on the class of the desired antibody. For IgM, because of its large molecular weight, it can be easily isolated by size-exclusion

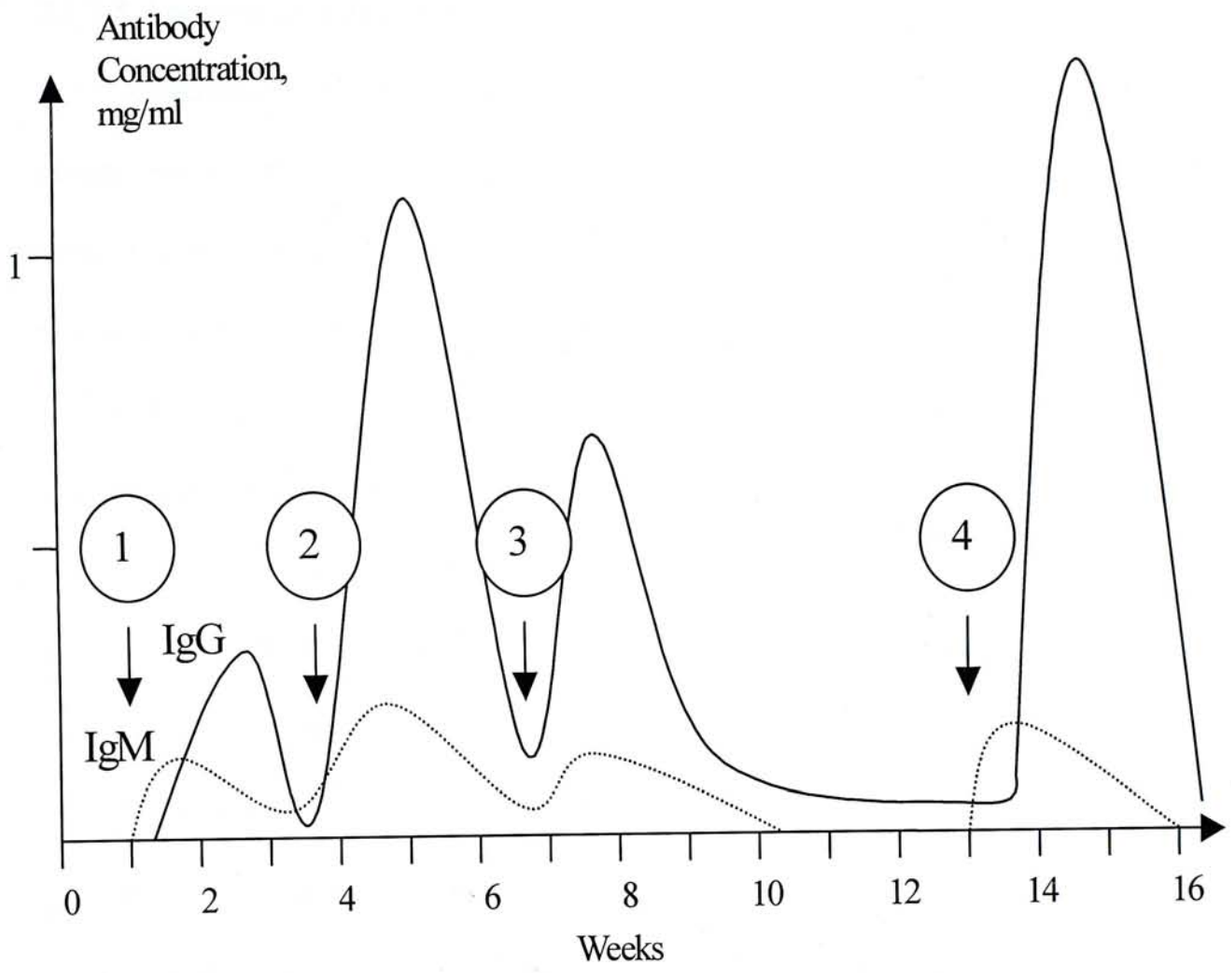


Figure 2.1 Immunogenicity and antibody production

1. A primary immune response to a soluble antigen induces first an IgM antibody production
2. A booster injection induces a marked increase in the IgG antibody level
3. A second booster injection soon after the previous one (3 weeks) is less effective in IgG antibody production
4. An increased delay (6 weeks) between two successive boosts yields more antibodies

(From Paraf, A., and G. Peltre. 1991. *Immunoassays in Food and Agriculture.* Kluwer Academic Publishers, Dordrecht, The Netherlands.)

column. Hereafter we shall describe the commonly used methods for IgG purification.

2.1.3.1 Ammonium sulfate precipitation

Ammonium sulfate precipitation is a classical method for subfractionating serum protein. While it may give some purification, it is mainly a method for concentrating antibody. In general, antibodies are precipitated between 40 to 60% ammonium sulfate saturation. The precipitated antibody can then be redissolved in phosphate buffer saline (PBS) and dialyzed before any further manipulations. Excess ammonium sulfate will interfere with most of these further purification procedures.

2.1.3.2 DEAE and QEAE Sepharose

These are positively charged matrices designed to remove negatively charged albumin, which will be the major contaminant in the antibody preparations. DEAE and QEAE are available attached to fast-flow matrices such as Sephadex, Sepharose, or acrylamide bead. In addition, FPLC columns containing similar matrices (MonoQ columns) are now available. IgG molecules bind poorly to such matrices and elute first in a gentle salt gradient while negatively charged albumin stays on the column. The advantage of this step is low-cost removal of the bulk of the albumin without exposing the antibody to extremes of pH.

2.1.3.3 Protein A and Protein G

Protein A is the method that has found widest acceptance in antibody purification. In most cases, other steps such as ammonium sulfate or DEAE are unnecessary. Protein A is a 42-kDa protein found on the cell walls of *Staphylococcus*

aureus, which binds to the Fc region of a wide range of antibody subclasses from many species (Langone 1982). While crude preparations of *S. aureus* may be used, protein A is generally coupled to various matrices such as Sepharose for use in column chromatography as this method is more convenient than using centrifugation to remove the cell/immunoglobulin complex. However, protein A binds poorly to the mouse IgG_1 subclass, the human IgG_3 subclass, and most rat monoclonal antibodies. To overcome this problem, protein G was introduced. Protein G is a 30- to 35-kDa protein isolated from the cell wall of β-hemolytic *streptococci* of the C or G strains (Kronvall 1973). Less is known about protein G than about protein A, primarily because protein G was not purified until 1984 (Bjorck and Kronvall 1984). Many antibodies from species or subclasses that do not bind well to protein A bind very well to protein G. Table 2.2 summarize the affinity of protein A and G for a spectrum of antibody sources.

2.1.3.4 Affinity chromatography

Affinity chromatography is generally used for isolating polyclonal antibodies from serum. This approach can be divided into two basic types, positive and negative. Negative selection is more common in which the protein suspected to be cross-reacting is immobilized on a gel matrix. The contaminating antibody then binds onto the column. The desired antibody, without cross-reaction to the immobilized ligand, will appear in the throw-through.

In the case of positive selection, the analyte is bound onto a solid matrix and the antibody sample is passed over the immobilized analyte at neutral pH. After

Table 2.2 Binding affinity of Protein A and Protein G to Fc regions of various immunoglobulin. S, strong affinity; W, weak affinity; W/S, weak overall affinity but much greater than that for Protein A; NB, no binding.

Species	Immunoglobulin class/subclass	Binding affinity	
		Protein A	Protein G
Human	IgG	S	S
	IgM	W	NB
	IgD	NB	NB
	IgA	W	NB
	IgG1	S	S
	IgG2	S	S
	IgG3	W	S
	IgG4	S	S
Mouse	IgG	S	S
	IgG1	W	W/S
	IgG2a	S	S
	IgG2b	S	S
	IgG3	S	S
Rat	IgG	W	W/S
	IgG1	W	W/S
	IgG2a	NB	S
	IgG2b	NB	W
	IgG2c	S	S
Bovine	IgG1	W	S
	IgG2	S	S
Sheep	IgG1	W	S
	IgG2	S	S
Goat	IgG1	W	S
	IgG2	S	S
Rabbit	IgG	S	S
Guinea pig	IgG	S	W
Porcine	IgG	S	W
Horse	IgG	W	S
Dog	IgG	S	W
Chicken	IgG	NB	NB
Cat	IgG	S	W
Monkey (rhesus)	IgG	S	S

washing the column, irrelevant antibody is removed in the running buffer. Antibodies specific to the analyte are then eluted using high or low pH or chaotropic agents. One disadvantage of this method is that when a truly high-affinity antibody is in the sample, it will be extremely difficult to elute it without using conditions which may destroy the antibody or the immobilized ligand (Table 2.3).

2.2 Methods

2.2.1 Purification of HDL and LDL from human serum

Fasting serum samples were obtained from the Department of Chemical Pathology, Prince of Wales Hospital. Lipoproteins were isolated by ultracentrifugal flotation. To prevent lipoprotein degradation during ultracentrifugation, PMSF (0.015% final concentration) and EDTA (0.04%) were first added into the serum. Serum was centrifuged at 38,000 r.p.m. at 4°C for 24 hours in a Beckman 42.1 rotor. In this run, chylomicrons and VLDL floated to the top while other serum proteins and lipoproteins migrated to the bottom of tube. The layer of VLDL and chylomicron was removed and a salt solution was then added to raise the background density to 1.019 g/ml. This salt solution was made by dissolving 153 g NaCl, 354 g of KBr and 100 μg EDTA in 1 liter of water giving a density of approximately 1.33 g/ml. The amount of salt solution needed to be added could be calculated by the following equation:

$$V2 = V1\frac{D - D1}{D2 - D}$$

Where V1= initial volume of solution, V2= volume of heavy solution to be added, D= required density, D1= original density, D2= density of the heavy solution (the density of serum is assumed to be 1.006 g/ml). The mixture was then centrifuged at

Table 2.3 Elution of antibodies from immunoadsorbents

Elution conditions	Comments
Diethylamine, 0.05 M, pH 11.5	Compatible with deoxycholate; often the method of choice for membrane antigens
Glycine-hydrochloride, pH 2.2-2.8	Incompatible with deoxycholate. Poor recoveries and aggregation of some membrane
Sodium thiocyanate, 3.5 M	Traces will inhibit iodination and coupling with fluorecein; remove by extensive dialysis
Guanidine-hydrochloride, 6 M	Very strong denaturing; efficient eluant but may cause severe aggregation
Urea, 2-8 M, pH 7	Heating in urea causes carbamylation of protein
Magnesium chloride, 2-5 M	Incompatible with deoxycholate
Propionic acid, 1 M	More effective than HCl at same pH, possibly owing to slight detergent action. May cause denaturation and irreversible aggregation
1 M Ammonia, pH 11.5	
Ethylene glycol (50%, v/v), at pH 11.5	Polarity-reducing agent; disrupts hydrophobic interactions
Dioxane (10%, v/v), at acid pH	Polarity-reducing agent; disrupts hydrophobic interactions
Electrophoresis, isoelectric focusing	Avoids denaturing conditions; somewhat slow and cumbersome

38,000 r.p.m. at 4°C for 24 hours. The top layer was pipetted out and the density of the remaining infranatant was adjusted to 1.06 g/ml by adding salt solution. LDL with a density cutoff from 1.019 to 1.06 g/ml would float to the top after centrifugation at 38,000 r.p.m. at 4°C for 48 hours. HDL was also isolated by the same method using a density cutoff of 1.08-1.2g/ml. The lipoproteins collected were dialyzed overnight at 4°C against 150 mM NaCl, 1 mM EDTA and 0.02% NaN_3 (pH8).

2.2.2 Purification of apolipoproteins

Purified HDL and LDL were delipidated in 20 volumes of ice-cold ethanol-ether (v/v 3:1) overnight at 4°C by gentle rocking. The insoluble apolipoproteins were collected by centrifugation for 20 minutes at 720 g, 4°C. The organic phase was decanted and the protein pellet was washed once with ethanol-ether (v/v 3:1) and then twice with cold anhydrous diethyl ether. Apo-HDL was solubilized in 150 mM NaCl, 20 mM Tris, 1 mM EDTA, 0.02% NaN_3 and 6 M Urea (pH8). Apo-LDL was solubilized in 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, 0,02% NaN_3, 10% glycerol and 2% SDS (pH7.2). The traces of ether in the sample were removed by blowing a gentle stream of nitrogen over the sample.

ApoA-I was isolated by size chromatography on Sephacryl S-200. Delipidated HDL was applied onto a 1.6 x 100 cm column. Elution was carried out at 4°C in 150 mM NaCl, 20 mM Tris, 1 mM EDTA, 0.02% NaN_3 containing 6 M Urea (pH8). The fractions in the main peak were pooled and dialyzed against PBS at 4°C. The column flow rate was 10 ml/hour. The purity of the apoA-I preparation was checked by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

ApoB100 was isolated by size chromatography using Sepharose CL-4B. Delipidated LDL was applied onto a 2.6 x 100 cm column. Elution was carried out at room temperature in 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, 0.02% NaN_3 and 0.1% SDS (pH7.2). Fractions corresponding to the main peak were pooled and dialyzed extensively against PBS at room temperature in order to remove excess SDS. The dialysis was performed at room temperature because the SDS would precipitate at 4°C. The column flow rate was 8 ml/hour. The purity of the apoB100 preparations was checked by 5% gel SDS-PAGE. Protein concentration was determined by the method of Bradford (Bradford 1976).

The qualities of the purified apolipoproteins were examined by double immunodiffusion and immunoelectrophoresis using standard antiserum obtained commercially.

2.2.3 Immunization of rabbit with apolipoprotein A-I and B100

Four male New Zealand white rabbits (2-2.5 kg, two for apoA-I and two for apoB100) were housed in individual metal cages and allowed free access to rabbit chow and water. A 12-hour light and dark cycle was maintained in the holding area. For each rabbit, 0.5 mg apolipoprotein was dissolved in 0.5 ml PBS which was then mixed with 0.5 ml cFA. The water-oil mixture was then emulsified by mixing the solution a few dozen times between two syringes connected with a double-hub needle (Herbert 1973). The stability of the emulsion was tested by allowing a drop of the mixture to fall onto the surface of a beaker of water, which should remain as a discrete globule. A total of 1 ml of emulsion was injected in four subcutaneous sites on the back skin of the rabbit (0.25 ml for each site). Injections were repeated at a three-week interval using same amount and volume of antigen, but cFA was replaced

by iFA. Three days after the last booster (three boosters totally), blood sample was collected from the ear vein. Around 40 ml blood could be obtained from each rabbit. Blood was allowed to clot at room temperature for 2 hours then at 4°C overnight. Serum was obtained by centrifugation at 2,000 g for 10 minutes and was stored in 1 ml aliquots at -20 °C.

2.2.4 Enzyme-linked immunosorbent assay (ELISA)

The presence of specific anti-apoA-I and B antibodies in the antisera was determined by indirect ELISA. 200 µl of apolipoprotein (25 µg/ml) in coating buffer (15 mM Na_2CO_3, 34.8 mM $NaHCO_3$, pH9.6) was added to each well of a 96-well ELISA plate. The tray was capped and incubated at 37°C for 1 hour. The coating solution was emptied and the wells were washed three times with PBST (PBS with 0.1% Tween20) with 5 minutes between each wash. The empty sites were saturated by incubating with 200 µl 1% BSA in PBS at 37°C for 1 hour. Plates were then washed three times with PBST. Antiserum (100 µl) at various dilutions was added (initial dilution of 1:200 and serially two-fold diluted to 1:204800) and incubated at 37°C for 1 hour. The plate was washed five times with PBSMT (PBS with 0.1% Tween20 and 3% dry milk) in order to remove unbound materials. Detecting antibody (100 µl of rabbit anti-mouse IgG with alkaline phosphatase (ALP) conjugated, 3000-fold diluted from stock) was then added to each well and incubated at 37°C for 1 hour. After washing three times with PBSMT and two times with PBST, each well was incubated with 100 µl substrate solution pNpp (10 mM p-nitrophenylphosphate disodium salt in 0.1 M diethanolamine, pH10.3, containing 0.5 mM $MgCl_2$) at room temperature. Color was developed in the dark for 30 minutes and the absorbance at 405 nm was measured in an ELISA plate reader. The titer of

the antiserum was defined as the dilution of the antiserum which will give an absorbance of 0.5.

2.2.5 Purification of lipoprotein specific immunoglobulin from antisera

2.2.5.1 Salt fractionation

Saturated ammonium sulfate solution was prepared by dissolving 1000 g ammonium sulfate in 1000 ml distilled water at 50°C and allowed to stand overnight at room temperature and adjusted to pH7.2 with diluted ammonia solution. Antiserum was diluted two-fold with PBS and saturated ammonium sulfate solution was added slowly while stirring to a final concentration of 45% saturated (v/v). If this was done too quickly, 'hot spots' would form which would denature the antibody. The mixture was stirred for a further 20-30 minutes at room temperature to allow full precipitation of protein. The protein precipitate was centrifuged down at 3000 g for 30 minutes at room temperature. The supernatant was discarded and the precipitate was redissolved in a minimum volume of PBS. The dissolved protein was dialyzed against five changes of PBS (pH8.0) at 4°C and the protein concentration was determined (Lowry et al. 1951).

2.2.5.2 Purification of immunoglobulin by Protein A affinity chromatography

Any aggregates in the antibody sample were removed first by filtering through a 0.45 μm membrane. Before applying the sample to the protein A column (1 ml protein A-Sepharose), the column was equilibrated with 3 bed volumes of PBS. According to the description of the manufacturer, protein A-Sepharose could bind 4.5-9 mg immunoglobulin per ml of gel. The antibody sample was applied to the column without exceeding the maximum binding capacity of the gel. The unabsorbed

materials were washed out with five volumes of PBS or until no material appeared in the effluent. Excessive washing should be avoided if the interaction between the antibody of interest and the ligand was weak. The absorbed immunoglobulin was then eluted by three column volumes of elution buffer (0.1 M citric acid, pH3.0). The effluent was collected in fractions to which sufficient amount of neutralizing buffer (1.0 M Tris-HCl pH9, 80 μl per ml fraction) was added to bring the pH up to around 7. To regenerate the protein A column, the column was run through an alternate 5 ml cycle of 0.1 M sodium acetate, pH4.5, containing 500 mM NaCl and 0.1 M Tris, pH8.5 containing 500 mM NaCl. Finally the column was re-equilibrated with 0.1 M PBS before the next run. If the column was to be stored, the column was equilibrated with 10 ml 20% ethanol. By measuring absorbance at 280 nm, the effluent fractions with protein were analyzed by 12.5% SDS-PAGE. The fractions containing the immunoglobulin fractions were pooled and dialyzed against PBS (pH8) containing 0.2% NaN_3.

2.2.5.3 Isolation of specific antibody by lipoprotein-coupled affinity chromatography

Purified HDL or LDL were coupled onto cyanogen bromide (CNBr)-activated Sepharose 4B. 5.5 g CNBr-activated Sepharose 4B was weighed out and suspended in 1 mM HCl (1 g freeze-dried powder gives about 3.5 ml final volume of gel). The gel was supplied freeze-dried in the presence of additives, which could be washed out by 1 L of 1 mM HCl on a sintered glass filter. Lipoproteins with a equivalent of 136 mg of protein content were dissolved in 27.5 ml coupling buffer (0.1 M $NaHCO_3$ pH8.3 containing 0.5 M NaCl). The coupling ratio recommended by the manufacturer was 7 mg protein per ml gel dissolved in 1.43 ml coupling solution. The lipoprotein was mixed with the gel and the mixture was rotated end-over-end for

1 hour at room temperature. After washing away the excess protein by five volumes of coupling buffer, the gel was transferred to 0.1 M Tris-HCl to block the unreacted active groups. For complete removal of uncoupled lipoprotein, the column was washed with 4 cycles of alternative high and low pH buffer. Each cycle consisted of 0.1 M acetate at pH4.0 followed by 0.1 M Tris-HCl at pH8.0 each containing 0.5 M NaCl. This procedure ensured that no free lipoprotein remained ionically bound to the gel. The coupled gel was equilibrated in PBS (pH8.0) containing 0.2% NaN_3 before the first run.

The immunoglobulin purified by protein A affinity column was applied onto the lipoprotein-coupled Sepharose 4B (19.25 ml of gel volume). The unbound immunoglobulin was washed out with PBS (pH8.0) and the lipoprotein-specific immunoglobulin was eluted by 0.1 M citric acid (pH3.0).

2.3 Results

2.3.1 Purification of apoA-I and B100

When HDL apolipoprotein was fractionated by Sephacryl S-200 gel filtration (Figure 2.2), only one main peak was observed (peak I). By electrophoretic analysis (Figure 2.3), peak I showed a single band with an apparent molecular weight of 28 kDa corresponding to the size of apoA-I (Figure 2.4). From 100 ml human serum, 35 mg of apoA-I could be isolated. The reactivity of the purified apoA-I with standard goat-anti-human apoA-I serum was examined by double immunodiffusion (Figure 2.5). Precipitin line was observed between standard antiserum and purified apoA-I, proving that the purified protein was apoA-I. The intensity of the precipitin line, when compared with the positive controls, suggests the binding affinity of the purified apoA-I is comparable to standard apoA-I and native HDL.

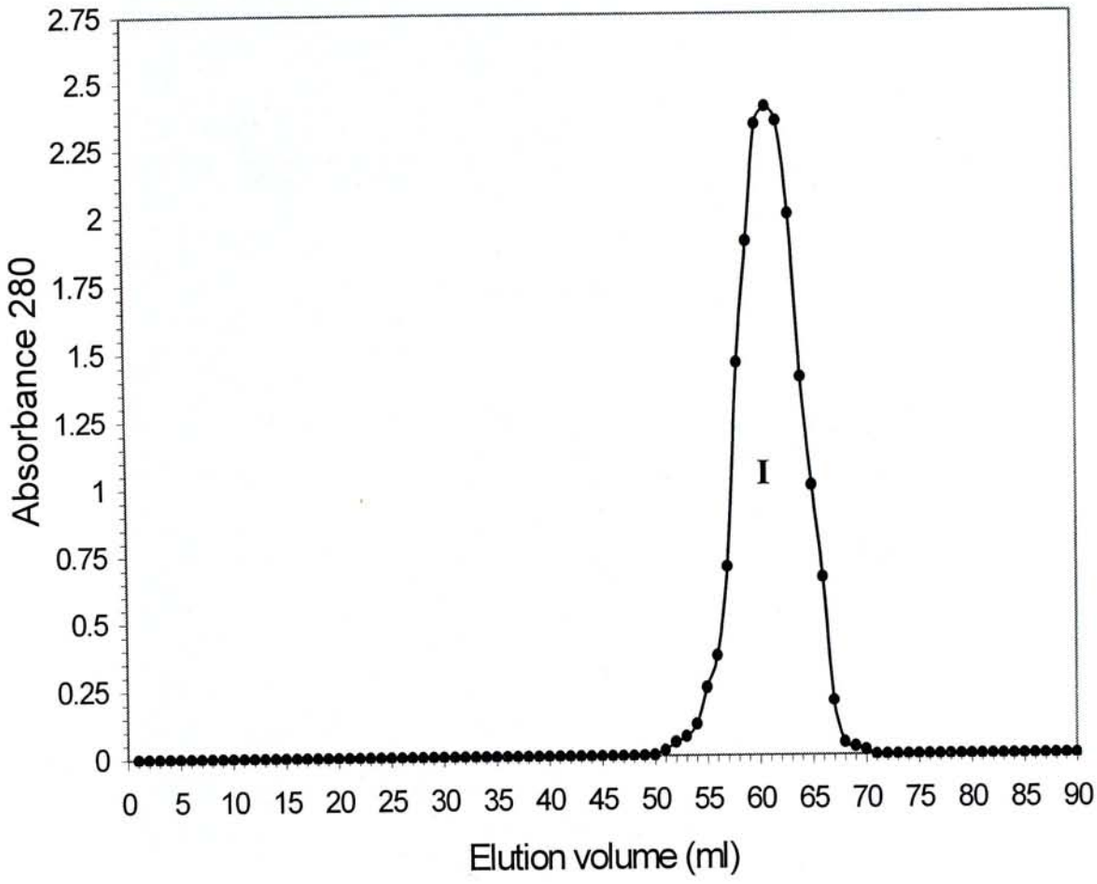


Figure 2.2 Sephacryl-S 200 gel filtration chromatography of HDL apolipoproteins. 10 mg of protein in 150mM NaCl, 20mM Tris, 1mM EDTA, 0.02% NaN_3 and 6M Urea (pH8) was applied and eluted at 10 ml/hr with the same buffer. The material from peak I was pooled for further analysis.

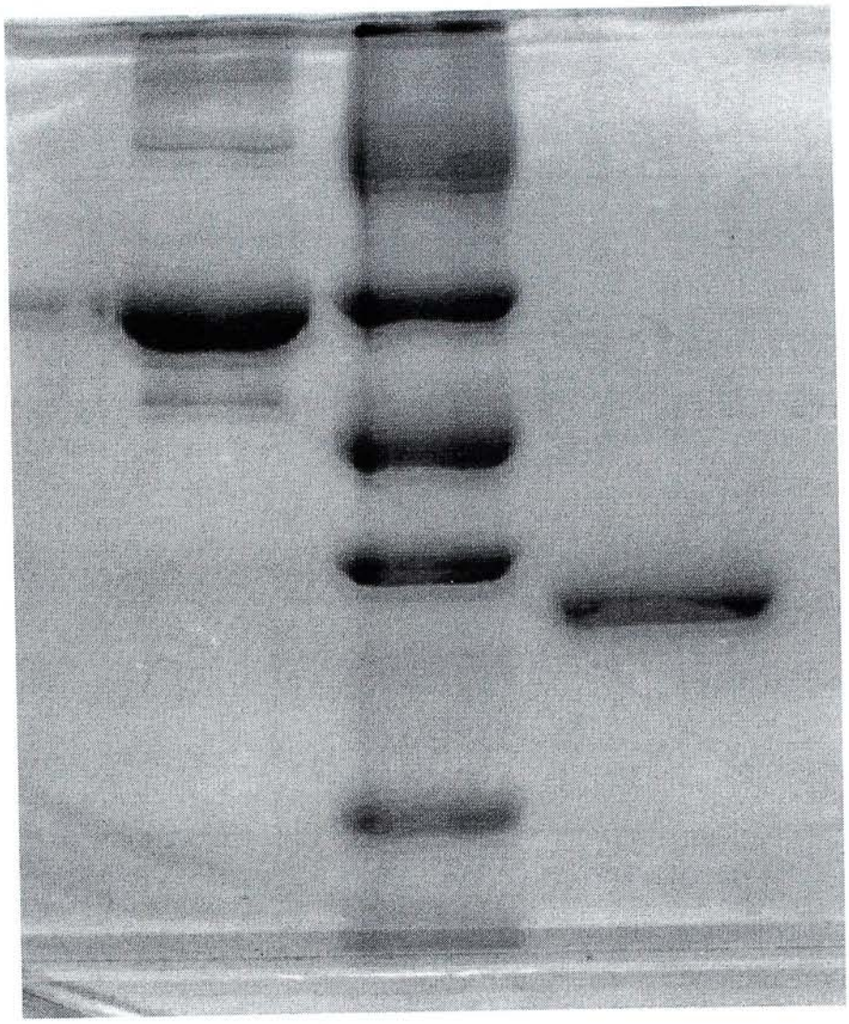


Figure 2.3 SDS-Polyacrylamide (12.5%, reducing) gel electrophoresis of ApoA-I. (A) Standard: albumin, 67 kDa; (B) Low molecular weight marker; (C) Peak I from the elution of apoHDL.

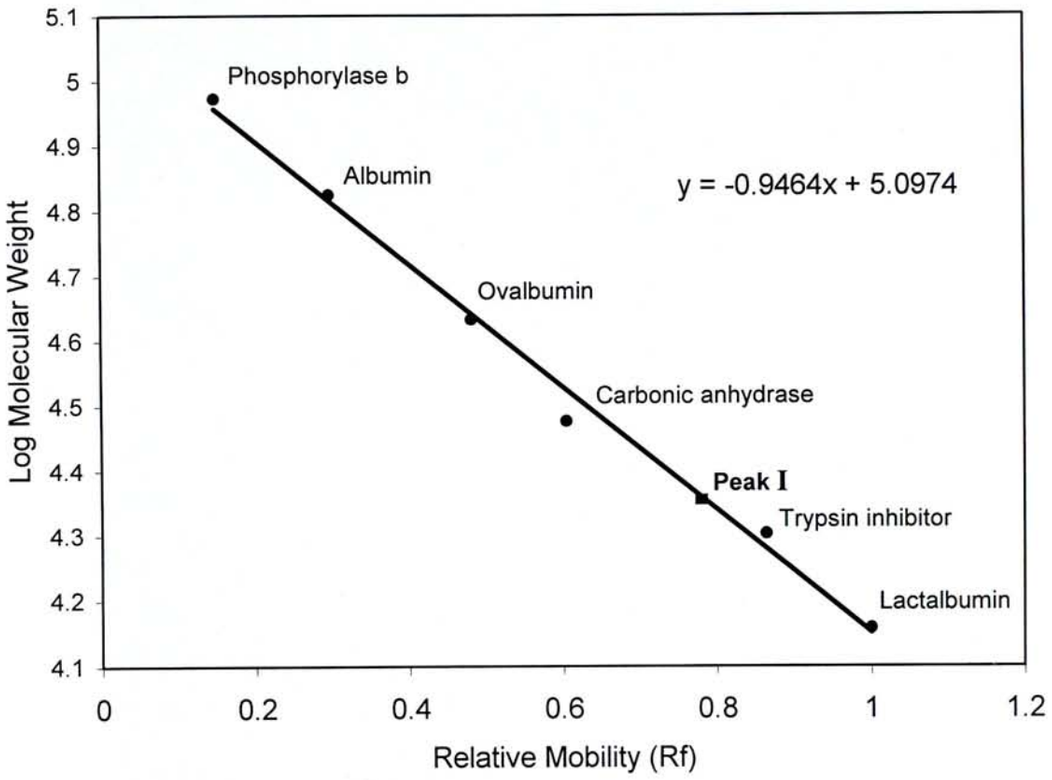


Figure 2.4 Standard curve for determination of protein molecular weight. Six references standards were run and their positions were plotted (●) and the linear best fit line was drawn. The Rf value for the purified protein (Peak I) then was calculated and plotted on the line (■), and the molecular weight was calculated.

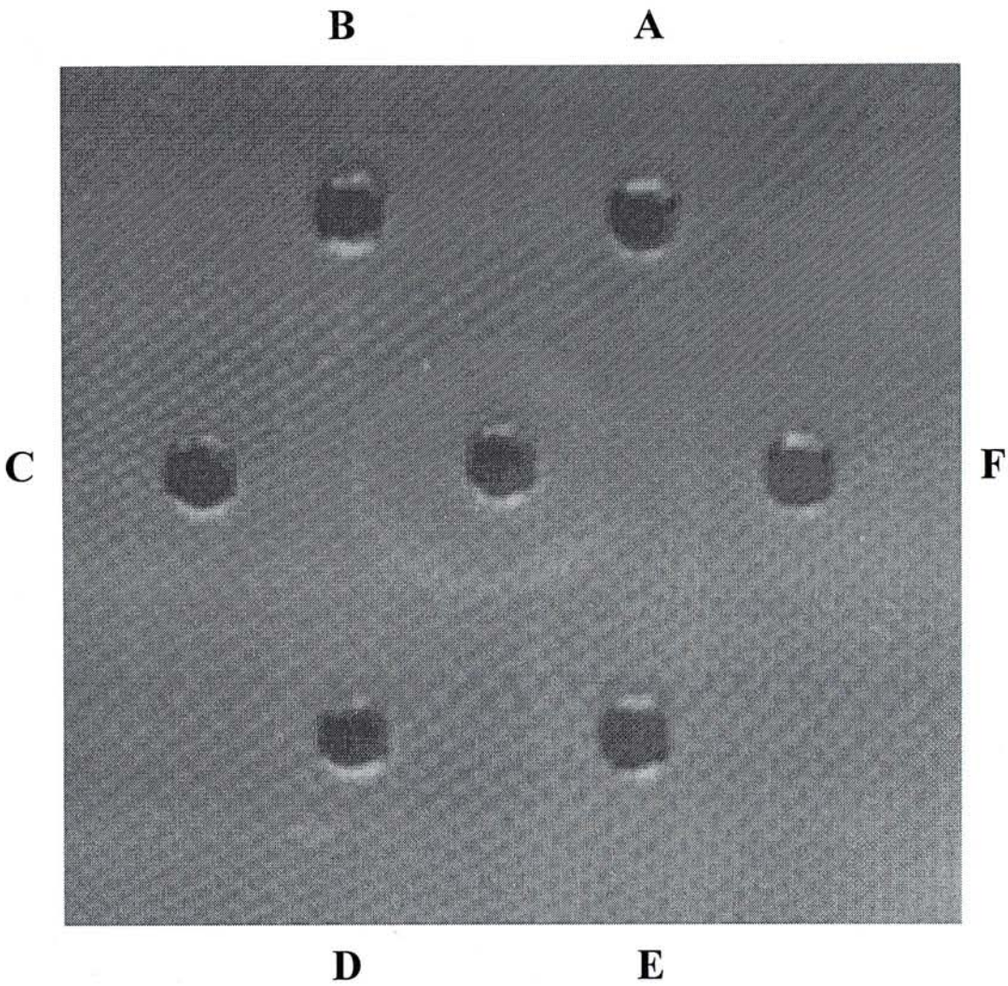


Figure 2.5 Double immunodiffusion of purified apoA-I, standard apoA-I and HDL with standard anti-apoA-I serum. The middle well contained 0.2 ml standard goat-anti-human apoA-I serum. Well A and E contained 10 μl of standard apoA-I (1 mg/ml); well C contained 10 μl of ten-fold diluted human serum; well B and D contained 10 μl of purified apoA-I (1 mg/ml); well F was the control containing PBS.

The elution profile of the LDL apolipoproteins fractionated by SepharoseCL-4B gel filtration chromatography is shown in Figure 2.6. A main peak (peak I) was observed. The material from peak I was pooled and further analyzed by SDS-PAGE. A single band was present with a molecular size similar to apoB100, i.e., 550 kDa (Figure 2.7). This pure protein was referred to as apoB100. From 100 ml human serum, 78 mg of apoB100 was purified. Immunoelectrophoresis was performed to examine the reactivity of the purified apoB100 to standard anti-apoB100 serum. From Figure 2.8, the precipitin line formed with standard antiserum proves that the purified protein is apoB100. The left shift of positive control LDL was due to the larger molecular weight of LDL than apoB100.

2.3.2 Purification of immunoglobulins from rabbit anti-apolipoprotein sera

Rabbit antisera against human apoA-I and apoB100 were produced as described in the method section. Their titers as determined by ELISA are shown in Figure 2.9 and 2.10. For anti-apoAI, an absorbance of 1.0 was obtained when the antiserum was diluted 1500-fold. Activity then gradually decreased with increasing dilution. The absorbance decreased to 0.5 when the antiserum was diluted to 55,000-fold which was defined as the titer of the antiserum. When it was diluted to 400,000-fold, the anti-apoA-I activity reached background level (absorbance 0.1). In the case of the anti-apoB100 serum, maximum antibody activity was observed at 700-fold dilution. Activity became undetectable when the dilution was increased to 400,000-fold. Using the same criterion as mentioned above, the titer of the anti-apoB100 serum was 60,000.

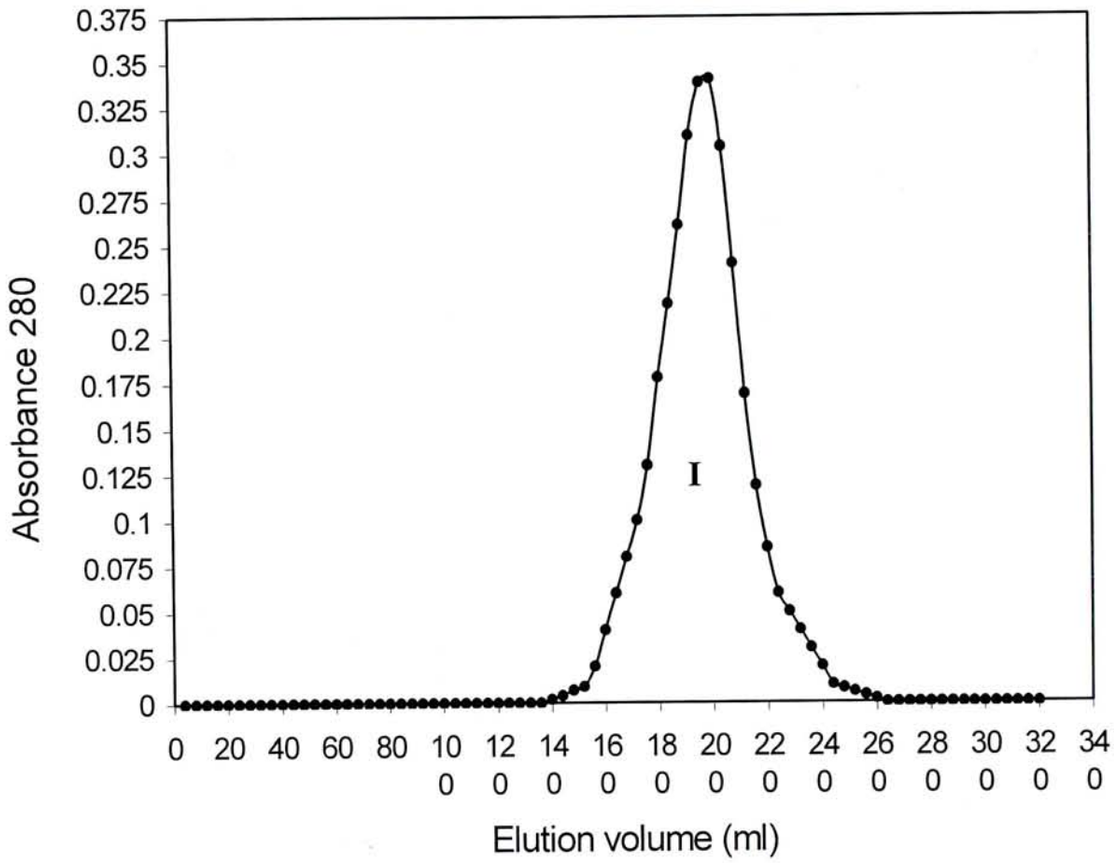


Figure 2.6 SepharoseCL-4B gel filtration chromatography of LDL apolipoproteins. 20 mg of protein in 150mM NaCl, 10mM NaH_2PO_4, 1mM EDTA, 0,02% NaN_3, 10% glycerol and 2% SDS (pH 7.2) was applied and eluted at 8 ml/hr with 150mM NaCl, 10mM NaH_2PO_4, 1mM EDTA, 0,02% NaN_3 and 0.1% SDS (pH7.2). The material from peak I was pooled for further analysis.

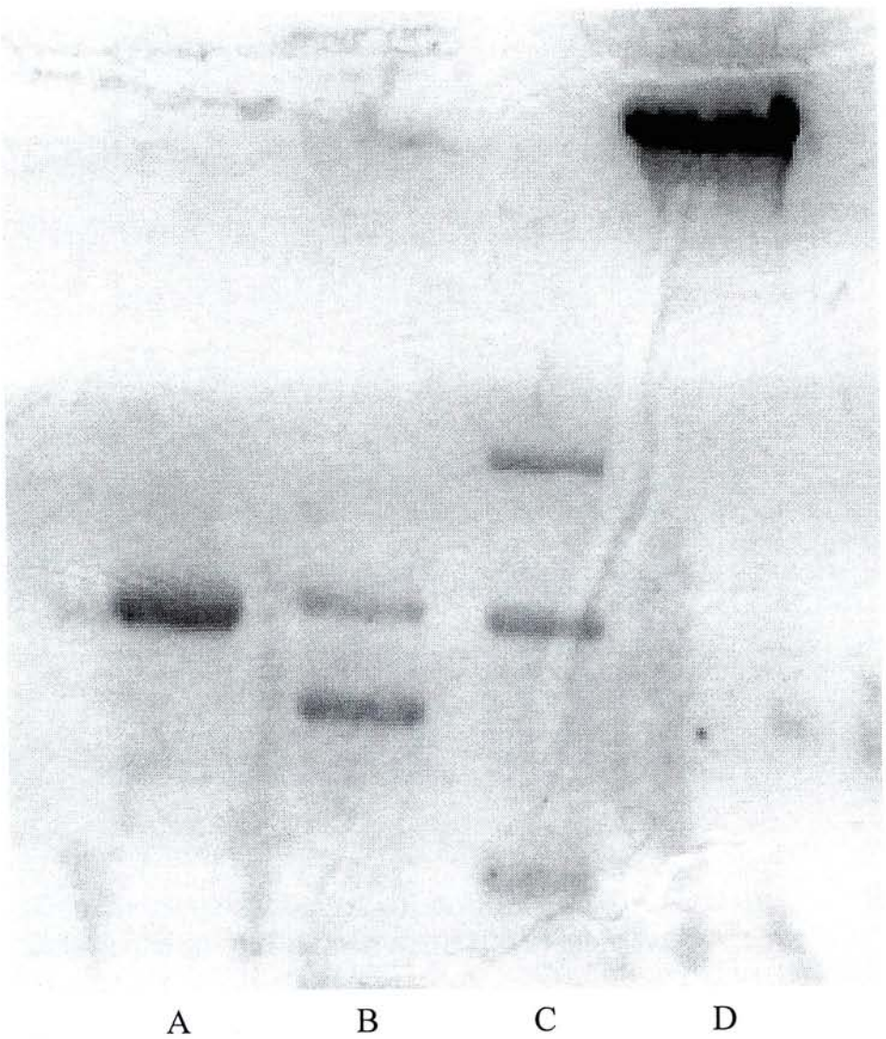


Figure 2.7 SDS-Polyacrylamide (5%, reducing) gel electrophoresis of peak I from apo-LDL separation. (A) Standard: albumin, 67 kDa; (B) High molecular weight marker; (C) Low molecular marker; (D) Peak I form the elution of aopLDL.

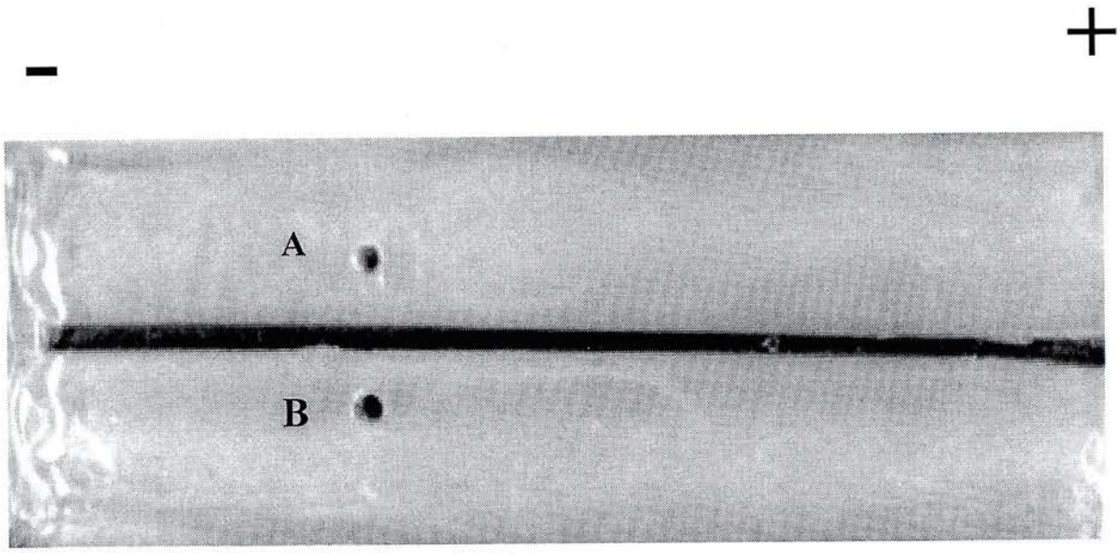


Figure 2.8 Immunoelectrophoresis of purified apoB100 and LDL with standard goat-anti-human apoB100 serum. Well A contained 10 μl of LDL (1 mg/ml) and well B contained 10 μl of purified apoB100 (1 mg/ml). The goat-anti-human apoB100 standard serum was placed in a trough cut in the 2% agarose.

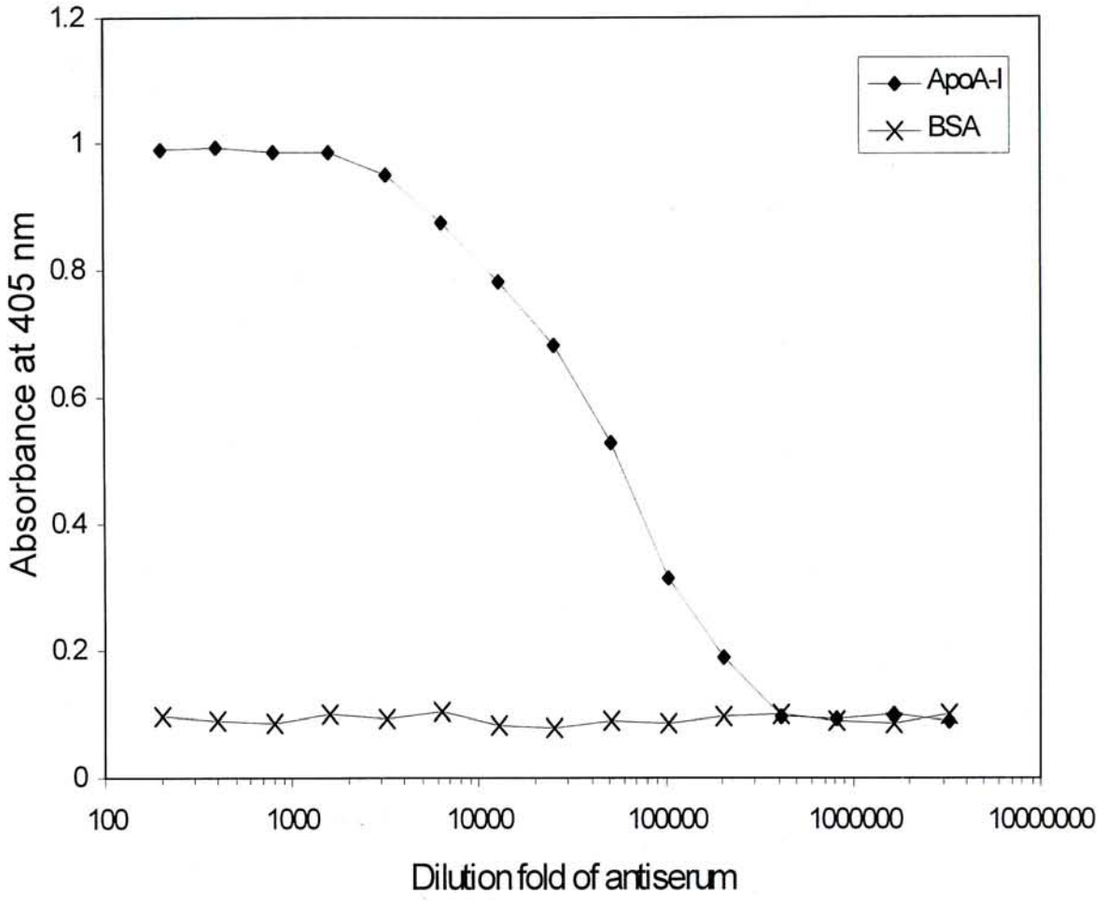


Figure 2.9 Anti-apoA-I activity in rabbit antiserum taken on day 3rd after the third booster. The control wells were pre-coated with BSA of same protein concentration.

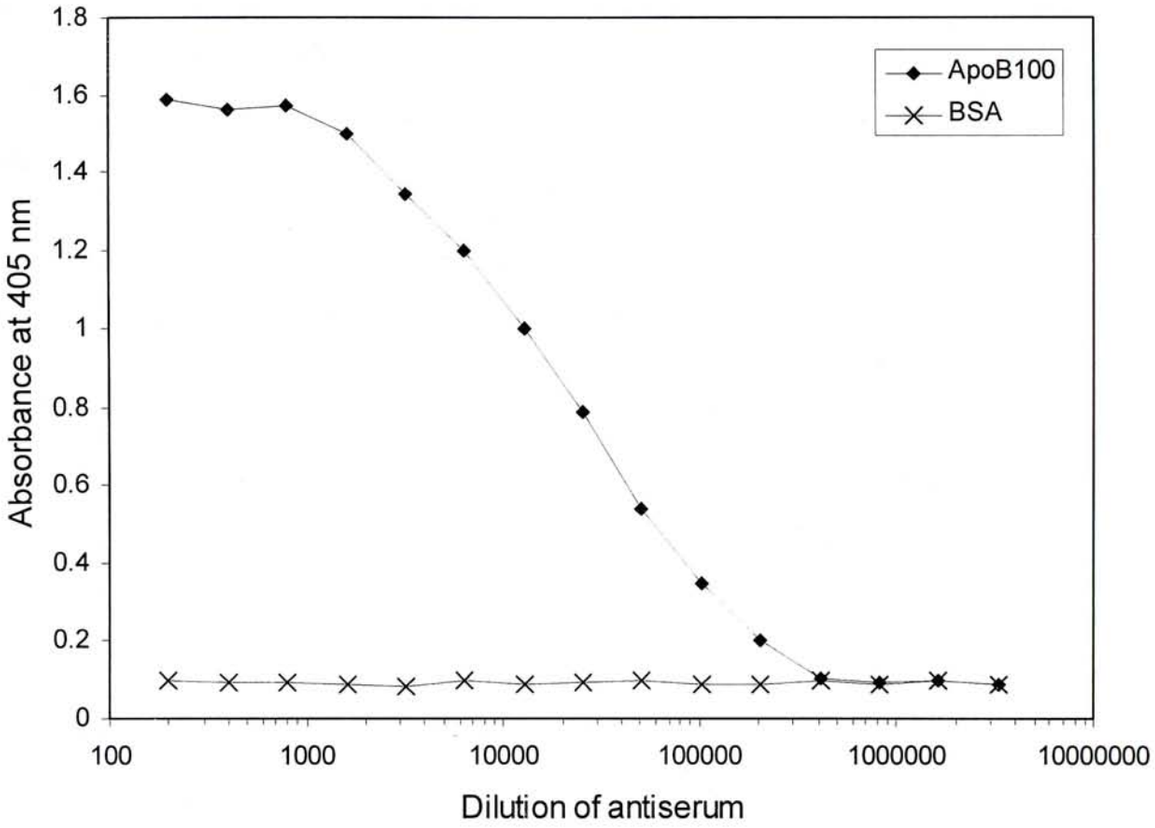


Figure 2.10 Anti-apoB100 activity in rabbit antiserum taken on day 3rd after the third booster. The control wells were pre-coated with BSA of same protein concentration.

From 10 ml anti-apoA-I serum, 125.3 mg of protein was extracted from the 45% ammonium sulfate precipitation. The protein was applied onto a protein A-affinity column and 80.3 mg of IgG was isolated. The IgG fraction was further purified on a HDL-coupled Sepharose 4B column and 2.08 mg of HDL-specific IgG was obtained (Figure 2.11). By protein determination of the samples before and after the HDL-affinity purification, it was estimated that some antibodies were retained in the column. These materials probably have a high affinity for HDL as they were not eluted by the 0.1 M citric acid. In order to elute these high-affinity materials, stronger reagents were used. 4 M magnesium chloride (pH7.2), 3.5 M sodium thiocyanate, 6 M urea (pH7) were tried and could not elute any of these high-affinity antibodies out, but dioxane (10%, v/v) could elute approximately 200 μg protein. By 12.5 % SDS-PAGE analysis, these proteins contained some IgG together with some other protein contaminants (Figure 2.12).

From 10 ml anti-apoB100 serum, 132 mg of protein was obtained after 45% ammonium sulfate precipitation. From this precipitate, 85.6 mg of pure IgG and 1.27 mg LDL-specific IgG were purified (Figure 2.13). The 12.5 % SDS-PAGE analysis of the purified polyclonal antibodies is presented in Figure 2.14. Two bands appeared in the antibody samples with the apparent molecular weight of 55 kDa and 25 kDa. These are presumably the heavy chain and light chain of the antibody molecule, respectively.

2.4 Discussion

Apolipoprotein A-I and apolipoprotein B100 are two of the main proteins in human serum. Their normal concentrations are both around 120 mg per 100 ml serum. In this study, both apoA-I and B100 were isolated from human serum. Using

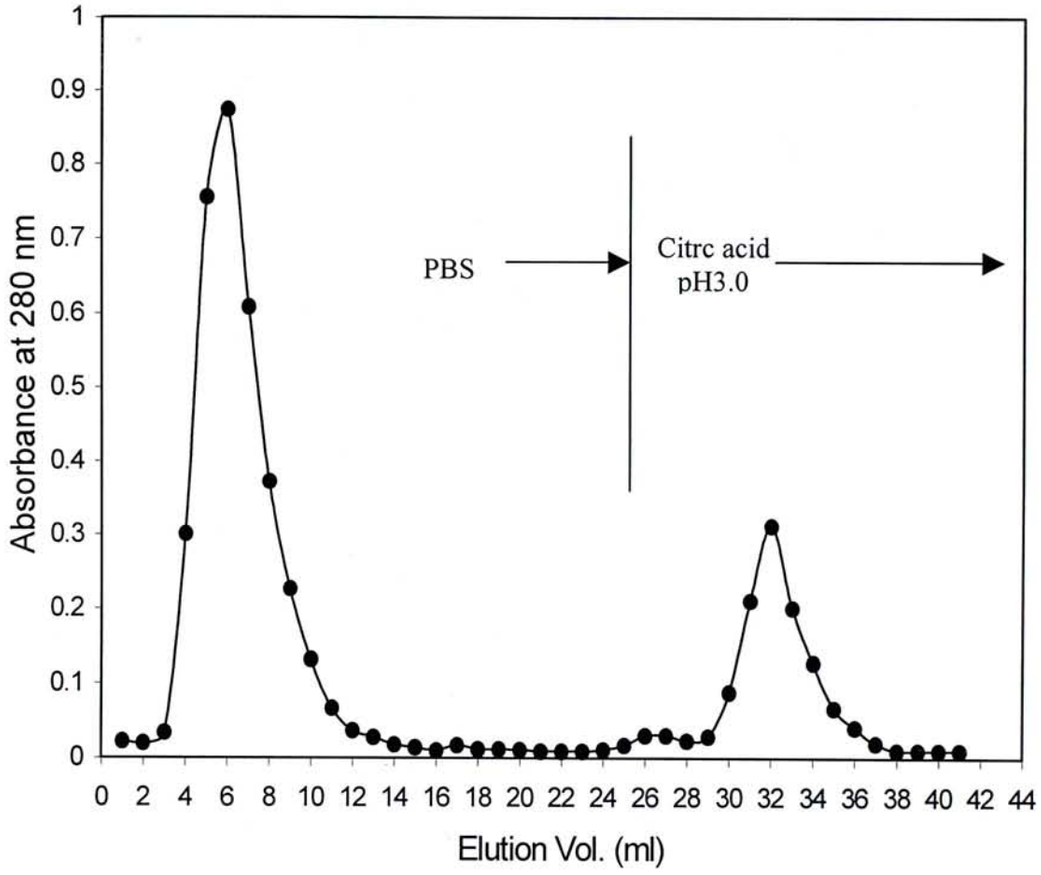


Figure 2.11 Purification of HDL-specific IgG from anti-apoA-I serum on HDL-coupled Sepharose 4B. The material eluted by citric acid was pooled for SDS-PAGE analysis.

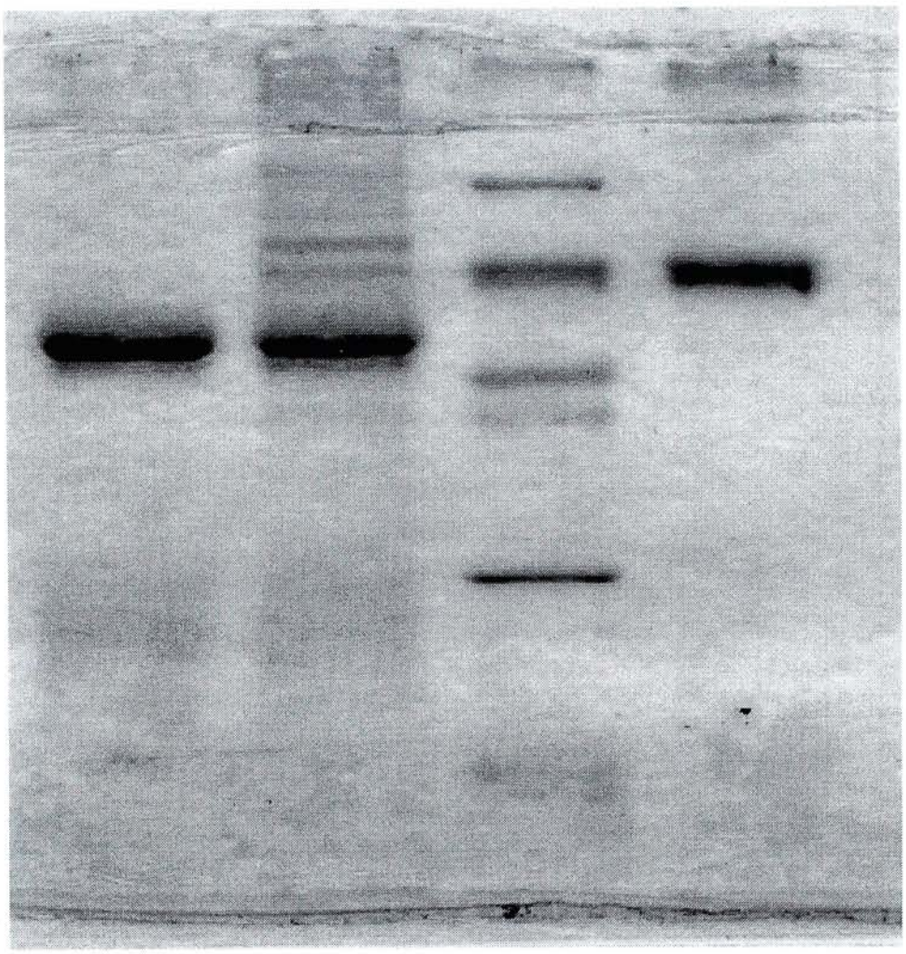


Figure 2.12 SDS-Polyacrylamide (12.5%, reducing) gel electrophoresis of proteins eluted by 10% dioxane. (A) proteins eluted by 0.1 M citric acid; (B) proteins eluted by 10% dioxane; (C) low molecular weight marker; (D) albumin.

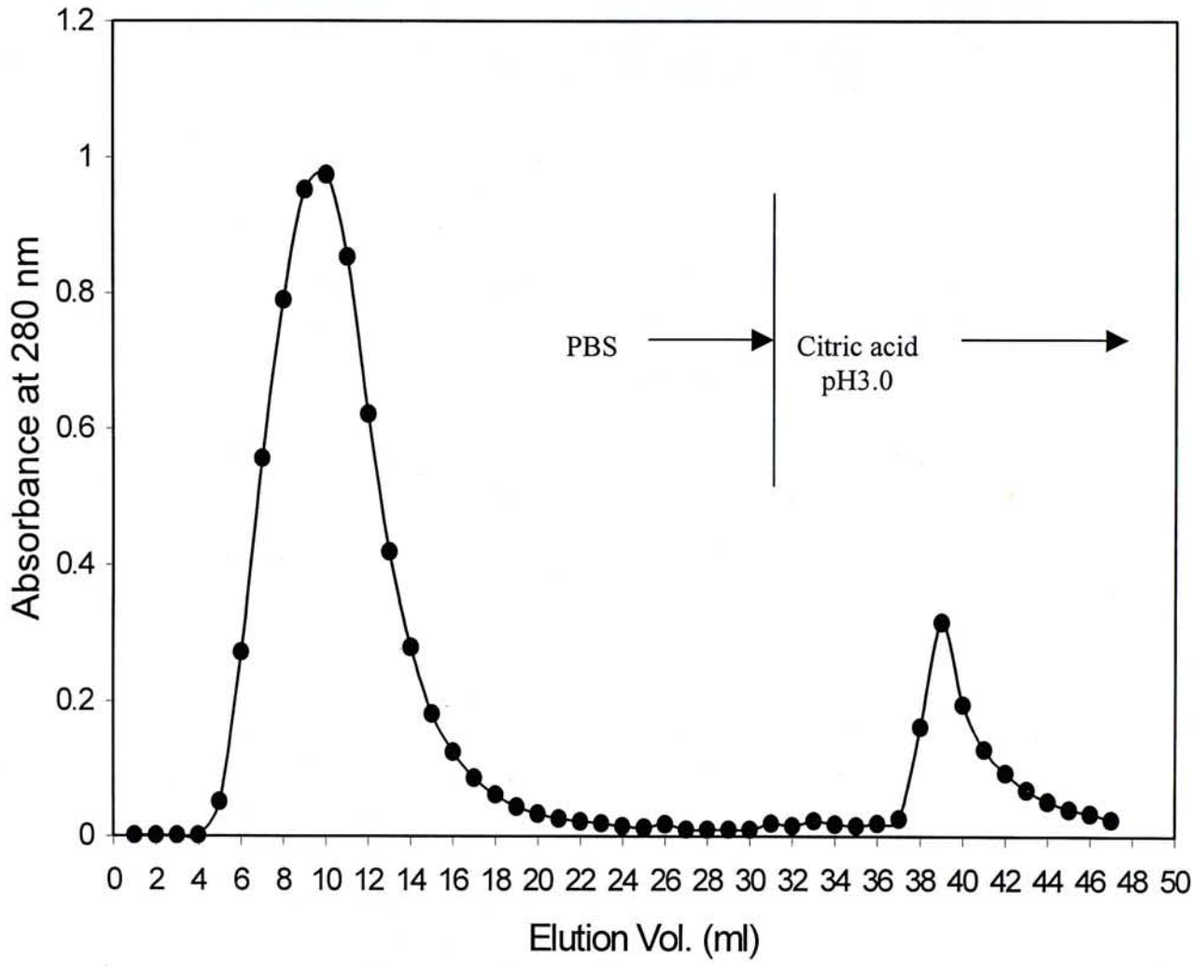
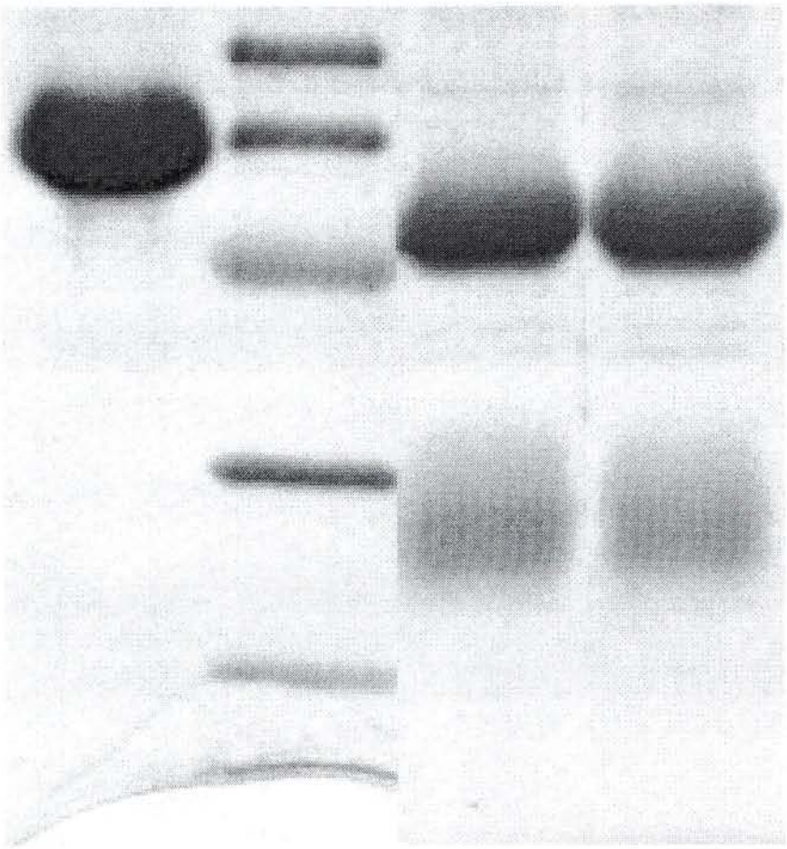


Figure 2.13 Purification of LDL-specific IgG from anti-apoB100 serum on LDL-coupled Sepharose 4B. The material eluted by citric acid was pooled for SDS-PAGE analysis.



A B C D

Figure 2.14 SDS-Polyacrylamide (12.5%, reducing) gel electrophoresis of purified rabbit polyclonal antibodies. (A) Standard: albumin, 67 kDa; (B) Low molecular weight marker; (C) HDL-specific antibody; (D) LDL-specific antibody.

100 ml human serum as starting material, 35 mg apoA-I and 78 mg apoB100 were obtained. The yield percentage, presuming 120 mg per 100 ml as the original concentration, was 29.17% for apoA-I and 65% for apoB100. The low percentage of apoA-I indicated a certain amount of apoA-I was lost.

Ultracentrifugation is not an ideal method for isolating lipoproteins. The loss of apoA-I from HDL during ultracentrifugal isolation has been well documented. In these reports, the loss of apoA-I was found to be about 30% (Curry et al. 1976; Fainaru et al. 1976; Mahley and Holcombe 1977) and reached as much as 50% (Fainaru et al. 1975). The loss of apoA-I from HDL by other isolation methods, such as precipitation and gel filtration chromatography (Rooke and Skinner 1979; Jahani et al. 1980), appears to be smaller. These findings suggest that some specific problems may be associated with ultracentrifugation.

Several factors unique to ultracentrifugation that could facilitate the loss of apoA-I from HDL have been suggested. They are wall effects (rotor configuration and tube type), high ionic strength of the ultracentrifugation medium, low temperature during ultracentrifugation, and ultracentrifugal force. Wall effects occur when the floating or sedimenting particles contact the centrifuge tube while traveling along their radial paths. When this happens, the impact or chemical interaction along the tube surface may cause the loss of apoA-I from HDL. Conventional systems of isolating lipoproteins by ultracentrifugation involve the addition of inorganic salt to the ultracentrifugal solvent to increase background densities. This addition of salt greatly increases the ionic strength of the solution. High ionic strength could be responsible for the loss of apoA-I from HDL. When lipoproteins are cooled, as during an ultracentrifugal run, the lipid components can undergo phase changes (Small et al. 1980). The structure of the lipoprotein can change at 4°C and thus could

cause a loss of apoA-I from HDL. Lastly, during ultracentrifugation, the sedimenting or floating particles are subjected to the high pressure generated in the fluid column by centrifugal force. ApoA-I at an air-water interface can be driven into aqueous solution by increasing surface tension (Shen and Scanu 1980). Since increasing the hydrostatic pressure acting on HDL particles may be equivalent to increasing the surface tension on the surface monolayer, this may be another mechanism which is responsible for the loss of apoA-I during ultracentrifugation. Some researchers have shown that apoA-I, once free in solution, will self-associate, and that self-associated apoA-I is slower to reassociate with lipid (Massey et al. 1981; Osborne and Brewer 1977). This would tend to enhance the separation of apoA-I from HDL. If monoclonal antibody specific to apoA-I is available, it is a better way to purify apoA-I by affinity chromatography, otherwise ultracentrifugation remains to be the most popular method.

One of the difficulties in apoB100 purification is the protein's lability. In a structural study of apoB100, the N-terminal (residues 1 to ~1,000) of apoB100 molecule was found to consist almost exclusively of trypsin-accessible peptides which was exposed on the surface of the LDL molecule (Yang et al. 1986). In order to inhibit the activity of trypsin, 0.015% PMSF (a serine protease inhibitor) was added to the serum. With the pre-treatment of PMSF, no proteolysis of apoB100 was found (Figure 2.7). If the treatment with PMSF was eliminated, several bands would appear in the SDS-PAGE analysis with smaller molecular weights (Figure 2.15). Serine protease-mediated proteolytic digestion is suggested to be the main cause of apoB100 degradation observed in other studies.

A B C D

Figure 2.15 SDS-Polyacrylamide (5%, reducing) gel electrophoresis of apoB100 purified with and without the pre-treatment of PMSF. (A) low molecular weight marker; (B) apoB100 purified with the pre-treatment of PMSF; (C) apoB100 purified without the pre-treatment of PMSF; (D) albumin.

In the elution profiles of apo-HDL and apo-LDL, only one main peak was observed. HDL and LDL contain 20 to 30% of proteins other than apoA-I and apoB100. HDL contains apoAII, apoE and apoC. LDL also contains apoC and apoE. The apparent lost of the other apolipoproteins in our chromatographies may be due to the dilution of these minor components in gel filtration chromatography. Moreover, due to the small size of these apolipoproteins, i.e., 6-8 kDa, they may be lost at the end of the chromatographic runs.

The major contaminant of antibody purification from serum is albumin. In normal serum, 60% of the protein content is albumin. After 45% ammonium sulfate precipitation of anti-apoA-I and anti-apoB100, nearly all albumins were removed (Figure 2.14). The purification efficiencies of the HDL- and LDL-specific antibodies can be estimated by assuming the normal IgG concentration in rabbit serum is 10 mg/ml and the percentage of antigen-specific antibody is 5% to the total IgG. Assuming these, the calculated purification efficiency is 41.6% for HDL-specific antibody and 25.4% for LDL-specific antibody.

There are several factors that contribute to the success of an immunoaffinity purification. The most crucial is the affinity of the antibody to the antigen.

The affinity of the antibody to the antigen is the most worrisome of the problems encountered with immunoaffinity purification. The ideal antibody is one that has a high affinity to the antigen and whose binding can be reversed by a simple and gentle change in an easily manipulatable variable such as pH. The binding of antibody to antigen takes place by the formation of hydrogen bond, electrostatic or coulombic interaction, van der Waals force, or hydrophobic interaction. A high-affinity antibody is likely to interact with its antigen by some or all of these non-covalent bonds. To elute this antibody, low pH buffer is not enough. In this study,

more extreme conditions were needed (10% dioxane) to elute the high-affinity antibody from the HDL-coupled affinity column. It indicates that the antibody and HDL are held together not only by electrostatic interaction but also the hydrophobic interaction. In this case, the 10% dioxane not only disrupted the hydrophobic interaction between the antibody and antigen but probably also damaged the solid phase bound HDL. Apolipoproteins are bound onto lipoproteins mainly by electrostatic and hydrophobic interaction. The amphipathic helix is a secondary structural motif found commonly in apolipoproteins (Segrest et al. 1974). This is a α-helix with opposing polar and nonpolar face. The polar face of the helix is exposed to the aqueous solution and interacts with the polar head of the phospholipid electrostatically. The nonpolar face of the helix is buried into the lipid layer and is held by the alkyl chains of phospholipid through hydrophobic interaction. If both of these forces are disrupted, the apolipoproteins will be dissociated from the lipoproteins.

Chapter 3

Production of monoclonal antibodies against apolipoprotein A-I and B100

3.1 Introduction

3.1.1 What is monoclonal antibody?

The monoclonal antibody era began in 1975 with a report in *Nature* by Köhler and Milstein entitled 'Continuous culture of fused cells secreting antibody of pre-defined specificity' (Köhler and Milstein 1975). They reported that they developed a technique to fuse the B-spleenocyte and myeloma (plasmacytoma) cell together to produce offspring cells with the desirable characteristics of both parent cells. The myeloma cell provided the genes for continued cell division in culture, whilst the B-spleenocyte provided the functional immunoglobulin genes. The cell line with these properties was called hybridoma. A monoclonal antibody may be defined as a uniform and homogeneous antibody directed against a single epitope or antigenic determinant, and produced continuously from one cell clone.

In this regard, monoclonal antibodies produced by a single clone of B cells have four distinctive advantages over antiserum as an analytic reagent. (1) they have uniform affinity and specificity in binding, (2) the antibodies are homogeneous, (3) monoclonal antibody can be produced in unlimited quantities, and (4) it obviates the need of a pure immunogen for immunization because selection of antibody can be performed during the production process.

3.1.2 The basic methodology

The basic principles involved in the production of monoclonal antibodies are illustrated schematically in Figure 3.1. The process involves five major steps: immunization, fusion, selection, cloning, and production.

3.1.2.1 Immunization of host

Mouse, rat, and human are the only three main animal species used for monoclonal antibody production. It is not possible to make hybridomas from outbred animals such as rabbits, sheep, and goats, as suitable myeloma lines are not available from these animals. The mouse is usually the preferred species, because (1) it is convenient to raise, (2) suitable mouse myeloma lines are available, and (3) the life cycle of the mouse is relatively short (gestation time 21 days; first mating at 6-8 weeks). BALBc mice are the animals of choice because there are several compatible myeloma lines.

3.1.2.2 Cell lines required for fusion

The cell line used for fusion must be immortal and it should be a myeloma (or plasmacytoma) cell, e.g., P3-X63-Ag8-653, Sp2/0-Ag14 (Sp2), and P3/NS1-Ag4-1 (NS-1). In myeloma cell, the endoplasmic reticulum system is well established which will enable them to secrete large amounts of antibody. On the other hand, it is better to select a myeloma cell line which cannot secret its antibody, otherwise its heavy chain and light chain will hybridize with those of the B cell, as the result, various types of hybrid antibody molecules are present with the desired specific antibody.

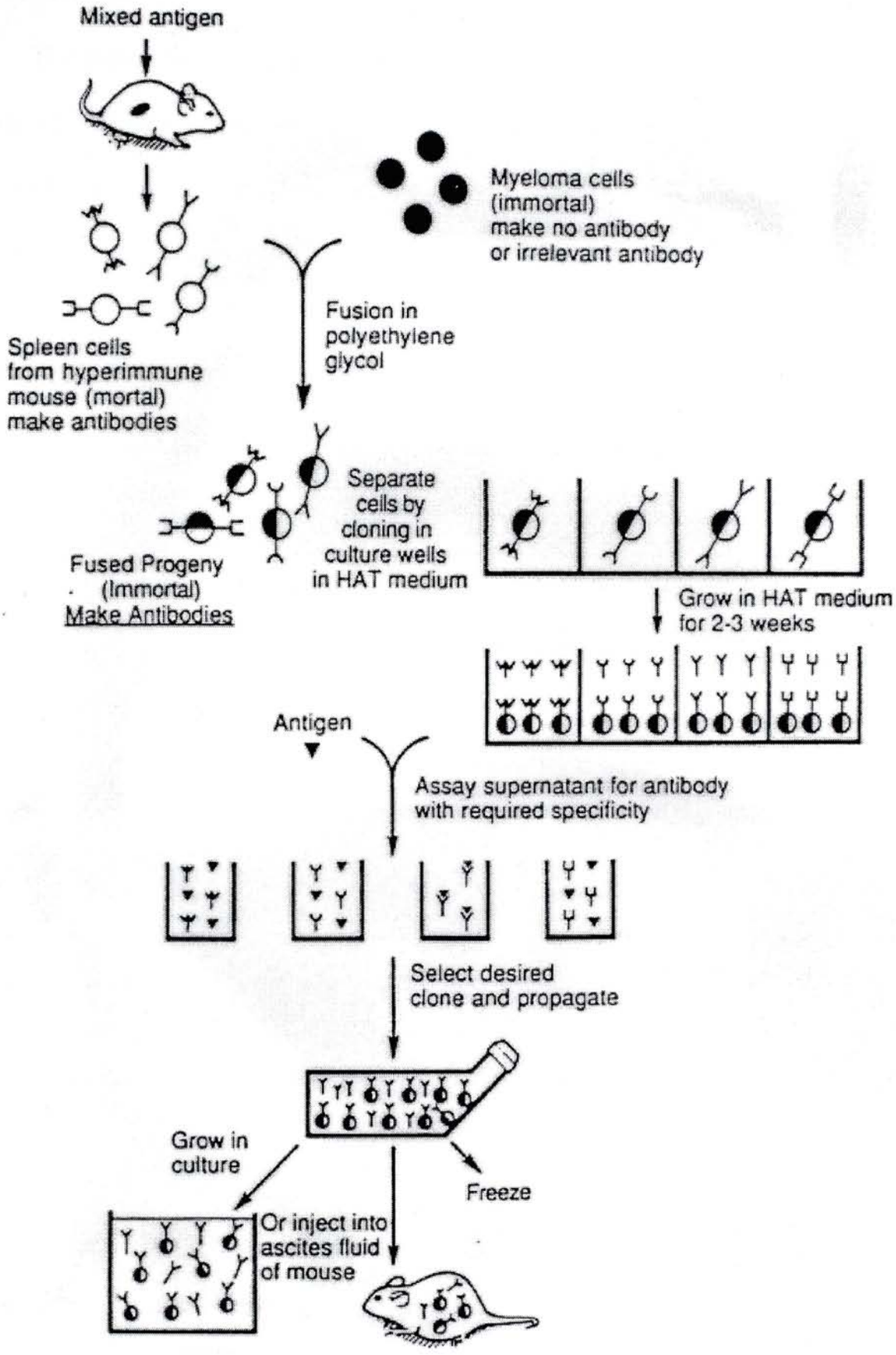


Figure 3.1 The general procedure for monoclonal antibody production. Spleen cells from immune mice are fused with HGPRT- myeloma cells using PEG. The cells is grown in HAT medium. Hybrids are tested for production of antibody of the desired specificity, and cloned by limiting dilution. The established clone is grown in culture or injected into mice for bulk production of monoclonal antibody.

3.1.2.3 Fusion

The fusion of cell membranes occurs at very low frequencies when cells are brought into close contact with each other but this fusion rate can be increased by the use of substances called fusogens. The original fusogen used was Sendai virus but this has now been superseded by polyethylene glycol (PEG) (Pontecorvo 1975),

$$HO(CH_2CH_2O)nCH_2CH_2OH \qquad n = 6\text{-}60$$

PEG is a hydrophilic compound which can remove the hydration shell from the cell surface. At 50% PEG (w/v), all water molecules in a solution becomes associated with PEG. The lack of cell surface water favors hydrophobic interaction between membrane lipids and cell fusion occurs between adjacent cells (Wojcieszyn et al. 1983). PEG may be obtained in molecular weight range from 200 to 20,000. It is toxic to cells and low molecular weight PEG is more toxic than high molecular weight PEG. Most successful fusions have been performed with PEG in the molecular weight range of 600-6000 (Davidson et al. 1976; Kelbe and Mancuso 1981). PEG concentration below 30% hardly produces any hybridoma, however, over 50% toxicity becomes overwhelming. The optimal concentration of PEG was found to be 50% (Galfrè and Milstein 1981). Both fusion frequency and cytotoxicity increase with time of exposure to PEG. Lower concentration of PEG (30-35%) can be tolerated by the cells for a longer time (7 minutes) than higher concentration (50% for only 1-2 minutes) (Gefter et al. 1977). Nevertheless, in most established protocols, a high concentration of PEG (50%) and a short period of exposure (1 minute) are used (Lane 1985). The pH of the PEG solution has also been shown to affect fusion frequency (Sharon et al. 1980). Maximal numbers of hybridoma are obtained at pH8.0-8.2. The speed at which the PEG is added to induce fusion does not appear to be too critical. Many published protocols suggest the use of PEG at

37°C, successful fusions have also been reported at 4°C. The ratio of spleen:myeloma cells used for fusion can range from 10:1 to 2:1.

3.1.2.4 Selection of hybrids

When a cell mixture contains spleenocytes and myeloma cells is subjected to fusion, we would expect other types of fusions such as lymphocyte-lymphocyte or myeloma cell-myeloma cell, or even higher multiples of these. The most common selection procedure was devised by Littlefield in 1964 (Littlefield 1964). Littlefield's procedure depends on the fact that when the main biosynthetic pathway for guanosine is blocked by the folic acid antagonist aminopterin, there is an alternative 'salvage' pathway in which the nucleotide metabolites hypoxanthine or guanine can be converted to guanosine monophosphate via the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) (Figure 3.2). Cells lacking HGPRT die in a medium containing hypoxanthine, aminopterin and thymidine (HAT medium) because both the main and salvage pathways are blocked. However, a $HGPRT^-$ cell can be made to grow in HAT medium if it is provided with the missing enzyme by fusion with a $HGPRT^+$ cell. Selection of $HGPRT^-$ myeloma is performed by using the toxic base analogue 8-azaguanine or 6-thioguanine, which is incorporated into the DNA via HGPRT. Because the salvage pathway is not normally essential for cell survival, mutants that lack HGPRT will continue to grow while cells that possess HGPRT will die. The hybrids, fused by a $HGPRT^-$ myeloma cell and a $HGPRT^+$ B cells, can survive in HAT selection medium.

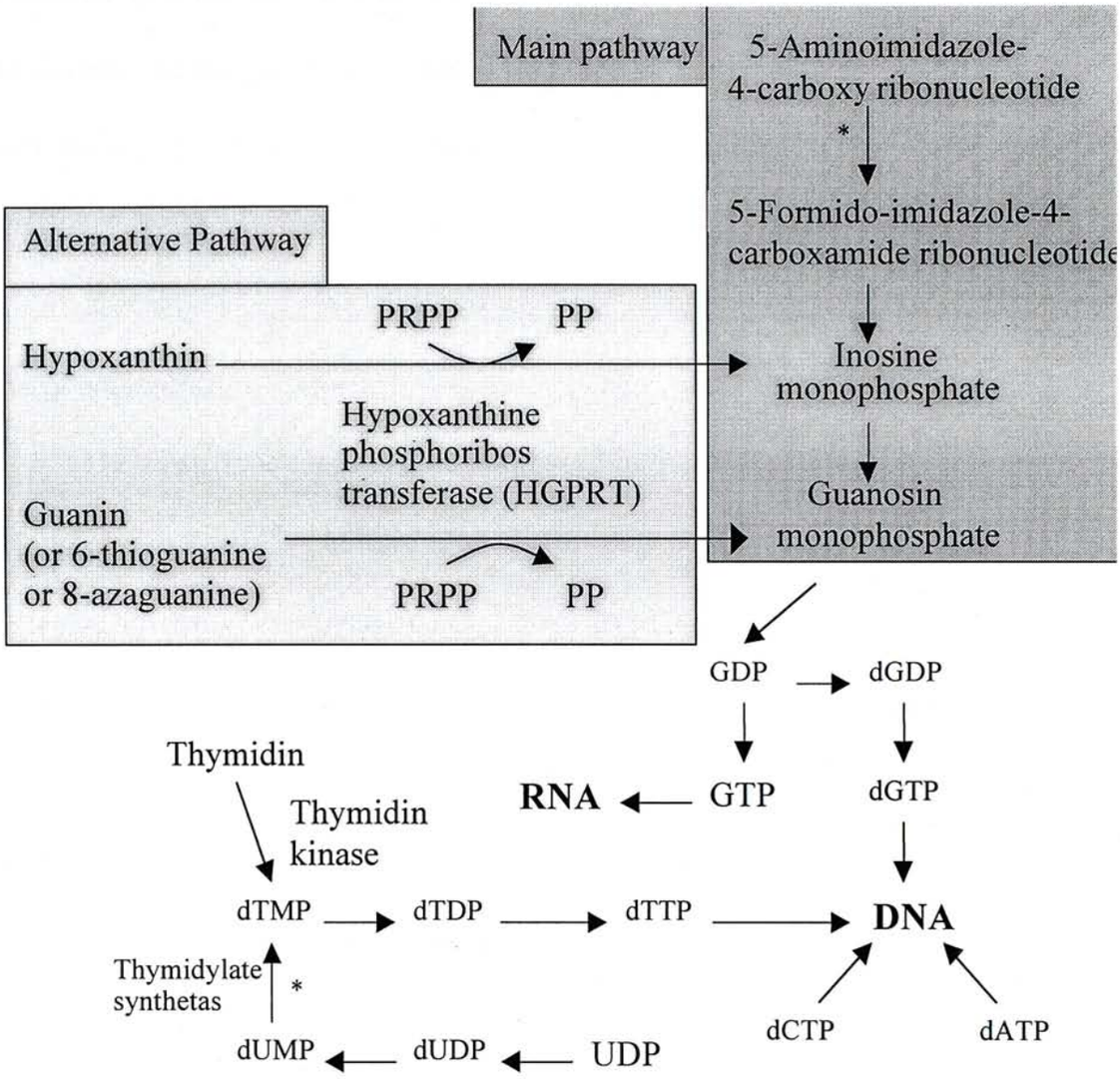


Figure 3.2 Metabolic pathways relevant to hybrid selection in medium containing hypoxanthine, aminopterin and thymidine (HAT medium). When the main synthetic pathways are blocked with the folic acid analogue aminopterin (*), the cell must depend on the 'salvage' enzyme HGPRT and thymidine containing the toxic base analogues 6-thioguanine or 8-azaguanine, which are incorporated into the cell via HGPRT. Only $HGPRT^-$ cells survive. $HGPRT^-$ cells cannot grow in HAT medium unless they are fused with $HGPRT^+$ cells.

3.1.2.5 Screening assay

As not all the clones can secrete the desired antibody, some of them are non-secretors or some secrete irrelevant antibody, a screening assay should be developed to screen out the positive clones. The practical constraints on screening assays are reliability, speed, cost and labor. For soluble antigens, the most popular and straightforward approach is ELISA (Kemeny 1991).

3.1.2.6 Cloning

The next issue that must be addressed in the production of hybridoma is that of clonality. The primary culture after fusion may contain many clones in each well. It is essential to separate the specific cells from those contaminating cells to ensure monoclonality. Limiting dilution is the most common cloning method (Lefkovits and Waldmann 1979). In this method, the cells are diluted to an average of one cell per well. If cells are grown in small numbers, the fraction of wells with growth should follow the Poisson distribution (Coller and Coller 1983):

$$f(0) = e^{-\lambda}$$

where $f(0)$ is the fraction of wells with no growth, and λ is the average number of clones per well. If $\lambda = 1$, $f(0) = 0.37$. To obtain a reasonable probability that wells with growth containing single clones, 37% of wells should have no growth. Hybridoma lines should be cloned by limiting dilution at least twice to make absolutely certain that each is a true clone. The major problem in this method of cloning is that the hybridoma cells cannot grow well at very diluted concentration. However, this can be overcome by using feeder cells. Feeder cells, such as thymocytes (Lernhardt et al. 1978), normal spleen cells (Galfrè and Milstein 1981)

and peritoneal cells (Hengartner et al. 1978), are good source of interleukin-6 which is an important factor to promote hybridoma growth.

3.1.2.7 Bulk production of monoclonal antibody

The most economical method for expanding clones is by *in vivo* infection. Injection of 10^6-10^7 hybrid cells into mice intraperitoneally will result in tumor formation after 2-4 weeks. Ten days before the injection of cells, the mice should be primed with 0.5 ml pristane (2,6,10,14-tetramethyl pentadecane) (Freund and Blair 1982). The antibody level in the ascites fluid can range from 2 to 10 mg/ml and 2 to 5 ml ascites fluid can be obtained from a mouse.

3.1.2.8 Monoclonal antibody purification

The methods available to purify polyclonal antibodies are also suitable for monoclonal antibodies purification (Table 3.1). The choice of the purification method is often governed by a number of variables, including the species in which it was raised, its class and subclass, the properties of the starting material. The main contaminants in ascites fluid are mouse serum proteins including substantial amounts of mouse albumin and also the mouse irrelevant IgG (Table 3.2).

3.2 Methods

3.2.1 Immunization of mice with apolipoprotein A-I and B100

For each antigen (apoA-I or apoB100), seven BALBc mice were immunized according to the following schedule: 100 µl PBS containing 15 µg of protein was emulsified with an equal volume of cFA and injected into each mouse intraperitoneally. Fourteen days later the mice were injected with a booster dose of

Table 3.1 Methods for purification of antibodies

Method	Examples
Fractional Precipitation	
Neutral salts	Ammonium sulfate, sodium sulfate, magnesium sulfate
Organic solvents	Ethanol, polyethylene glycol (PEG)
Metal ions	Zinc
Short-chain fatty acids	Caprylic acid
Organic cations	Rivanol
Electrophoretic Separation	
Carrier free media	Free boundary electrophoresis
Solid support	Zone electrophoresis on cellulose, acrylamide, or starch gel
Isoelectric focusing	Liquid media (sucrose gradient), gel media (acrylamide)
Ion-Exchange and Gel Filtration Chromatography	
Anion exchangers	Aminoethyl (AE-), diethylaminoethyl (DEAE-) and quarternary aminoethyl (QAE-) cellulose, Sephadex, or Sepharose
Cations	Carboxymethyl (CM-) cellulose, Sephadex, or Sepharose
Gel filtration	Sephadex G series, Sepharose 2B, 4B, 6B or CL 2B, CL 4B, CL 6B, Sephacryl S series, Biogel agarose or polyacrylamide beads, Ultrogel polyacrylamide-agarose beads
Preparative Ultracentrifugation	
	Sucrose density gradients, salt gradients
Affinity Chromatography	
	Protein A, protein G, protein A/G, thiophilic adsorption, avidin-biotin systems, antigen affinity column, anti-immunoglobulin affinity column, hydroxylapatite

Table 3.2 Sources for purifying antibodies

Source	Antibody type	Total antibody, mg/ml	Specific antibody, mg/ml	Contaminating antibodies	Possible purity of specific antibody
Serum	Polyclonal	10	1 at least (10% max)	Other serum antibodies	10% at best
Tissue Culture (in vitro techniques)					
With 10% FCS	Monoclonal	1	0.05% (5%)	Calf antibody	> 95%
Serum-free media	Monoclonal	0.05	0.05 (100%)	None	> 95%
Tissue Culture (in vivo techniques)					
Ascites	Monoclonal	1-10	0.9-9 (90%)	Mouse antibody	90%

35 μg of the protein by the same method but using iFA instead of cFA. Mice were totally boosted three times with a fourteen-day interval between each injection.

3.2.2 Preparation before fusion

Methods described under this section, unless otherwise specified, were carried out in a laminar flow cabinet. Tissue culture reagents were warmed to 37°C before use. Cell counts were determined in a hemocytometer. Cells were harvested by centrifugation at 1,000 r.p.m. for 5 minutes. Cell cultures were incubated at 37°C with an atmosphere of 5.0% CO_2.

3.2.2.1 Preparation of tissue culture working solutions

Fetal calf serum (FCS) was heat inactivated at 56 °C for 30 minutes in order to inactivate serum reactive proteins (e.g. complements) which could damage the cells in culture. FCS was dispensed in 25 ml aliquots and stored at -20 °C until to use.

Powder RPMI obtained from Gilbco was used as the basic culture medium. One liter of RPMI was prepared by dissolving a package of RPMI powder (1 Liter) in nano-pure water (700 ml). Sodium biocarbonate ($NaHCO_3$, 3.7 g) was added to the medium until fully dissolved. The pH of the medium was adjusted to 7.1-7.2 by either 1 N HCl or 1 N NaOH solution. Nano-pure water was then added to bring the volume to one liter. The medium was then sterilized by negative pressure filtration through a 0.22 μm pore size filter membrane. The sterilized medium, which is referred to a plain medium (P-RPMI), was dispensed into 500 ml air-tight bottles and stored at 4 °C prior to use.

Culture medium which contains FCS, antibiotics and other essential components is referred to as complete medium (C-RPMI). Unless otherwise

specified, all cells were cultured in C-RPMI (contained 10% heat inactivated FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and 300 μg/ml L-glutamine).

Hybridoma selective medium (HAT medium) was C-RPMI supplemented with sodium hypoxanthine (0.1 mM), aminopterin (0.4 μM) and thymidine (1.6 μM). Hybridoma proliferation medium (HT medium) was the same as HAT medium without the aminopterin.

3.2.2.2 Preparation of spleen cells

The mouse with the highest titer was chosen and boostered three days before fusion with 200 μl PBS containing 10 μg antigen intraperitoneally (Shibier et al. 1988). The immunized mouse was sacrificed by cervical dislocation and swabbed with 70% ethanol. The mouse was cut open to expose the spleen. The spleen was removed and placed in a 144 x 21 mm sterile Petri dish containing 5 ml C-RPMI. All fat was removed from the spleen and then it was transferred into a fresh dish containing 5 ml C-RPMI. A 21 G needle mounted on a 1ml syringe was used to tease the spleen to release the cells. All the released cells were transferred to a 10 ml centrifuge tube and tissue clumps were allowed to settle by standing the tubes for 5 minutes. The cell suspension was pipetted into a fresh 10 ml tube, without disturbing the tissue clumps. The suspension was made up to 10 ml with extra C-RPMI and the cells were centrifuged down. The plain supernatant was discarded and the cells pellet was resuspended in 10 ml of PBS. The cells were then transferred to a 50 ml culture tube and 10 ml sterile water was added to lyze the red blood cells. Immediately, 10 ml of 2X PBS was added and the cells were washed two times with P-RPMI. The spleenocytes were then resuspended in 20 ml C-RPMI and the viability of the cells was determined by trypan blue exclusion test: 50 μl cell suspension was diluted with

four volumes of PBS and mixed with 250 µl 0.2% trypan blue and the viable cell number was counted. Around 10^8 spleen cells was obtained per mouse.

3.2.2.3 Preparation of myeloma cells

The myeloma cell line used in the fusion was P3/NS1/1-Ag4-1 (NS-1). NS-1 cells are resistant to 10^{-4} M 8-azaguanine and will not grow in HAT medium. However, HAT sensitive myeloma may sometimes revert to HAT resistance due to mutation. The reverted cells would decrease the successful growth of the hybrids. In order to ensure HAT sensitivity, NS-1 cells were cultured in C-RPMI containing 8-azaguanine (20 µg/ml) for one week before fusion. Two 25 ml flasks of NS-1 with a cell density of 2-3 x 10^5 cells/ml were subcultured on the day before fusion and maintained at the exponential growth phase. Before fusion, a total of 10^7 NS-1 cells were collected and resuspended in C-RPMI. The cells were washed two times with P-RPMI and viability was determined by the trypan blue exclusion test.

3.2.3 Fusion

The spleenocytes collected from one spleen was mixed with myeloma cells in a 50 ml tube in a ratio of 10 spleen cells to 1 myeloma cell. The cell mixture was centrifuged and the tube was tapped to losen the cell pellet after removing the supernatant. The tube was put into a beaker of water at 37°C and 1 ml of pre-warmed PEG was added slowly over a period of 1 minute, with constant stirring using a pipette tip. It is critical that the cells must not be in contact with the concentrated PEG solution for more than 2 minutes, otherwise the cell membranes would eventually be disrupted. After adding the PEG, 1 ml of P-RPMI was immediately added over a period of 1 minute and then a further 4 ml of P-RPMI was added over

the next 3-4 minutes. The P-RPMI should be added slowly, without stirring, and down by the side of the tube. Then, a further 20 ml of P-RPMI was added following by 15 ml of C-RPMI with 20% FCS, again running it slowly down the side of the tube. The culture tube was inverted once and left at 37°C for 2 hours, allowing the cell membranes to stabilize. Finally, the cells were plated out in 96-well plates with 100 µl/well. The plates were incubated in the incubator at 37°C with 5% CO_2. After 24 hours, 100 µl of 2x HAT selection medium was added to each well. After 1 week, 100 µl of culture supernatant was removed from each well and replaced with HAT selective medium. Growth of hybrid colonies was visible microscopically after another several days of incubation (around 10 days after fusion). The growth of hybrids would turn the color of the medium to yellow due to the excretion of acidic metabolites. When the medium turned yellow, the culture supernatant was replaced by new HT medium.

3.2.4 Screening assay for positive clones

When the clone grew to cover one forth of the well, 100 µl of the culture medium could be screened to see if it is secreting the desired antibody. Screening was performed by standard indirect ELISA (Section 2.2.4)

3.2.5 Limiting dilution cloning

All the cells were harvested from the positive well and the cell number was counted. The hybridoma was cloned by limiting dilution in which the hybrid cells were grown in 96-well plate with a fixed cell density of 1 cell per well. Spleen cells from a normal mouse were used as feeder cells. They were prepared by the same method as described in the preparation of immunized mice spleenocytes. The feeder

cells were washed and resuspended in 4 ml C-RPMI. All dilutions were made by C-RPMI.

Assuming a hybridoma cell concentration of 10^5 cells/ml, a 20 µl of the suspension diluted to 2 ml would give a cell density of 1000 cells/ml. 200 µl of this hybridoma suspension was then mixed with 2 ml feeder cells, and diluted to 40 ml to give 5 cells/ml. The hybridoma-feeder cell mix was then plated into a in 96-well plate at 200 µl/well. This, in principle, will yield one hybrid cell per well. The cells were allowed to grow at 37°C in 5% CO_2. This would take about 2 weeks before the medium started to turn yellow. Wells with clones were screened by ELISA and positive clones were subcloned at least twice until all the clones in one plating become positive. The monoclonality is then be ensured at this point.

3.2.6 Determination of isotype

The isotype of the monoclonal antibody was determined by indirect ELISA using isotype-specific secondary antibodies (Section 2.2.4). These antibodies were specific to IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, IgA, κ-light chain and λ-light chain.

3.2.7 Cryopreservation of myeloma and established hybridoma cell lines

3.2.7.1 Freezing cells

Cryopreservation is necessary to preserve cell lines indefinitely. The cells were harvested, counted and centrifuged. The supernatant was discarded and the cells were resuspended in cold freezing mixture (90% FCS and 10% dimethyl sulfoxide, DMSO) with a cell density of $5x10^6$ cells/ml. 1 ml aliquots were transferred into the cryotubes and the freezing procedure was started: cells were left in cryotubes on ice for 2 hours, then the tubes were wrapped in insulated bubble film, packed in a

polystyrene box padded with cotton wool and placed in a -70°C freezer overnight. Finally the box were put into liquid nitrogen tank.

3.2.7.2 Thawing cells

Cells were thawed out rapidly in a water-bath maintained at 37°C. 10ml of C-RPMI was used to dilute the cells. It was important to dilute out the DMSO quickly as it would be toxic above 4 °C. The thawed cells were centrifuged down and resuspended in fresh C-RPMI.

3.2.8 Bulk production of monoclonal antibodies from ascites

The cloned hybridoma cell line was cultured *in vitro* in a large scale. At the same time, ten BALBc mice were prepared for innoculation by injection with 0.5 ml Pristane intraperitoneally. Seven days after the Pristane injection, the hybridoma cells were harvested, resuspended in sterile PBS at a concentration between $2x10^6$ to $2x10^7$ cells/ml and 0.5 ml of the cell suspension was injected intraperitoneally into each of the primed mice. The mice were inspected daily for swelling of the abdomen (indicating ascitic fluid production). As soon as there was considerable swelling or if the mouse was about to die, the mice were killed and the ascitic fluid was collected by drawing from a 19G needle fitted syringe. All the cells or fat tissues in the ascites fluid were removed by centrifugation.

3.2.9 Purification of monoclonal antibodies from ascites

The purification methods for polyclonal antibody (chapter 2.2.5) were used. The ascites was diluted two fold with PBS and the protein was precipitated at 45% (v/v) ammonium sulfate saturation. The precipitate was centrifuged down and

redissolved in PBS (pH8.0). The crude IgG preparation was dialyzed against PBS (pH8) at 4°C to remove ammonium sulfate. The antibodies were then purified by protein G-Sepharose CL-4B chromatography. PBS was used as the loading buffer and 0.1 M citric acid (pH3.0) as elution buffer. Neutralizing buffer (1.0 M Tris-HCl pH9.0) was also used to bring the effluent pH up to around 8 immediately after collection of the fractions.

3.2.10 Western blot analyses of the monoclonal antibodies

The specificities of AB6 and BE8 were analyzed by western blotting. 10 μg of apolipoprotein, lipoprotein and 1 μl of human serum were loaded on SDS-polyacrylamide gels. After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes (0.45 μm) and the membranes were subsequently blocked with PBSMT at room temperature for 2 hours. After washing with PBSMT, the membranes were incubated with purified AB6 or BE8 (0.3 μg/ml in PBSMT) at 4°C overnight. The membranes were then washed with PBSMT and incubated in ALP-conjugated rabbit anti-mouse antibody.

3.2.11 Iodination of apolipoproteins

50 μl apolipoprotein (400 μg/ml) dissolved in 0.25 M phosphate buffer (pH7.4) was mixed with 10 μl NaI^{125} (1 mCi). The mixture was then added to an iodogen pre-coated eppendorff tube (2 μg iodogen in 50 μl dichloromethane and evaporated dry). Iodination was performed at room temperature for 11 minutes and 0.5 ml PBS was added to stop the reaction. Free iodine was removed by Sep-pak column (C18 column). The reaction mixture was applied to the column and free iodine was

washed out by 50 ml 0.1% trifluoroacetic acid (TFA). The iodinated apolipoprotein was eluted with 5 ml 60% acetonitrile in 0.1% TFA.

3.2.12 Binding of the monoclonal antibody to iodinated apolipoprotein

Various concentrations of monoclonal antibody (50 μl) were incubated with 100 μl ^{125}I-labeled apolipoprotein (diluted with 1% BSA in PBS to give 20,000 c.p.m.) at 4°C overnight. 100 μl of Sac-cel (anti-mouse IgG antibody-bound cellulose bead) was pipetted into the mixture and was allowed to stand at room temperature for 30 minutes. One ml of distilled water was then added and the suspension was centrifuged for 5 minutes at 1,000 g. The supernatant was aspirated and radioactivity in the pellet was counted.

3.2.13 Competitive displacement analysis

50 μl of monoclonal antibody in a dilution which gave 50% precipitation of ^{125}I-labeled apolipoprotein was mixed with 100 μl of ^{125}I-labeled apolipoprotein (20,000 c.p.m.). 50 μl of various concentrations of unlabeled apoA-I, apoB100, HDL, LDL, VLDL, albumin or lipoprotein-depleted human serum (LPDS) were then added and incubated overnight at 4°C. Final precipitation with Sac-cel was carried out as described above. Maximum binding (B_{max}) was determined by replacing the unlabeled antigen with PBS. Normal mouse antibody instead of monoclonal antibody was used to determine the non-specific binding ($B_{n.s.}$). The degree of displacement was expressed as B/ B_{max}:

$$B/ B_{max} = \frac{B_{sample} - B_{n.s.}}{B_{max} - B_{n.s.}}$$

The value of B/B_{max} will fall within the range of 0 to 1. A smaller value represents a higher degree of displacement.

3.3 Results

3.3.1 Development of monoclonal antibodies

Seven mice were immunized with apoA-I and seven were immunized with apoB100. The mouse with the highest antibody activity was chosen for fusion (Figure 3.3 and 3.4).

2.3×10^7 spleenocytes obtained from the apoA-I immunized mouse were fused with 2.3×10^6 of myeloma cells. A total of 89 wells out of 384 wells containing growing hybridoma cells were observed. The fusion efficiency was 23.2%. Three of these (B6, D9, F2) scored positive upon initial screening by ELISA. During the limiting cloning of the anti-apoA-I clones, D9 and F2 lost their specific antibody activity and clone B6 was successfully cloned to monoclonality after two subcloning. In the second subcloning, 38.5% of the wells did not have hybrid growth and as calculated by the Poisson equation, the average number of clones per well was 0.95. Since all the hybrids in this round of subcloning were positive for anti-apoA-I activity, B6 was probably a homogeneous clone at this stage.

In the apoB100 fusion, 3.1×10^7 spleenocytes were fused with 3.1×10^6 myeloma cells. A total of 91 wells out of 384 wells showed hybrid growth. The fusion efficiency was 23.7%. Among these wells, five were screened to be positive; A5, B3, B7, E8 and H10. For the cloning of the anti-apoB100 clones, E8 was successfully cloned. The other four clones all lost their activities during subcloning. After three rounds of subclonings, the calculated average number of clones per well for E8 was 0.92 and all the hybrid clones showed anti-apoB100 activity. Thus, at

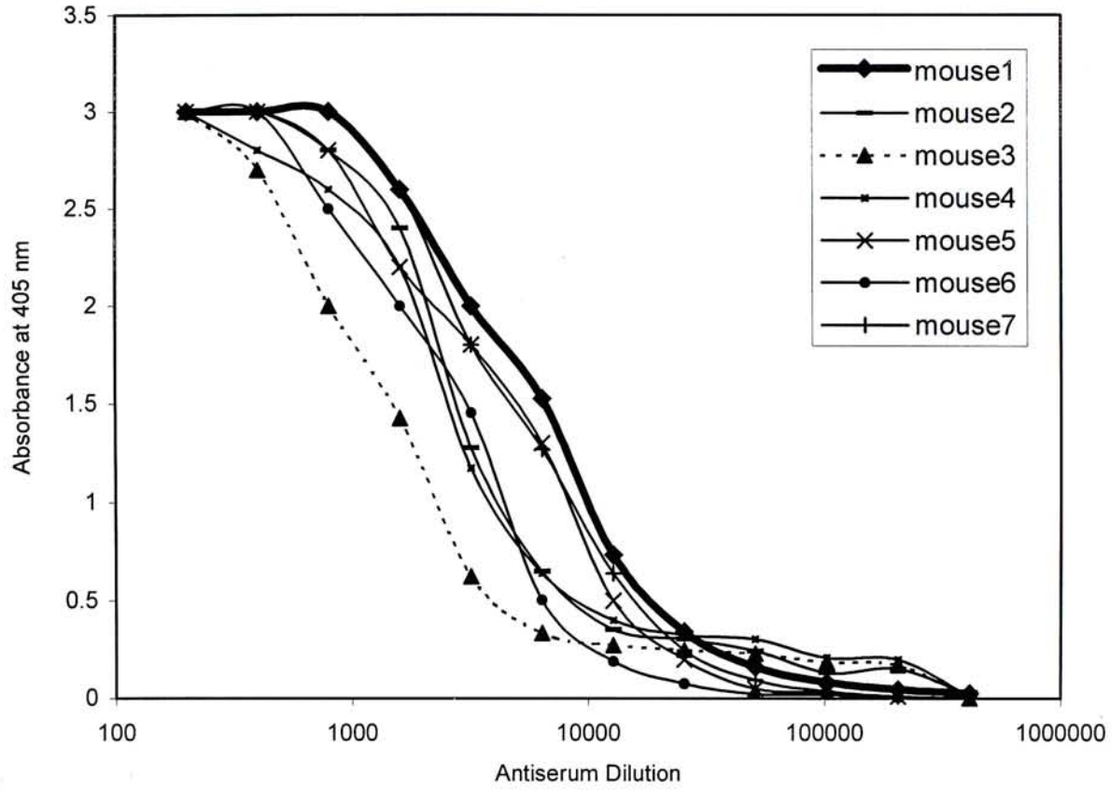


Figure 3.3 Anti-apoA-I antibody (IgG) activity in the sera taken on day 4 after the 3rd booster of apoA-I injection in seven mice. Anti-apoA-I activity was measured by ELISA.

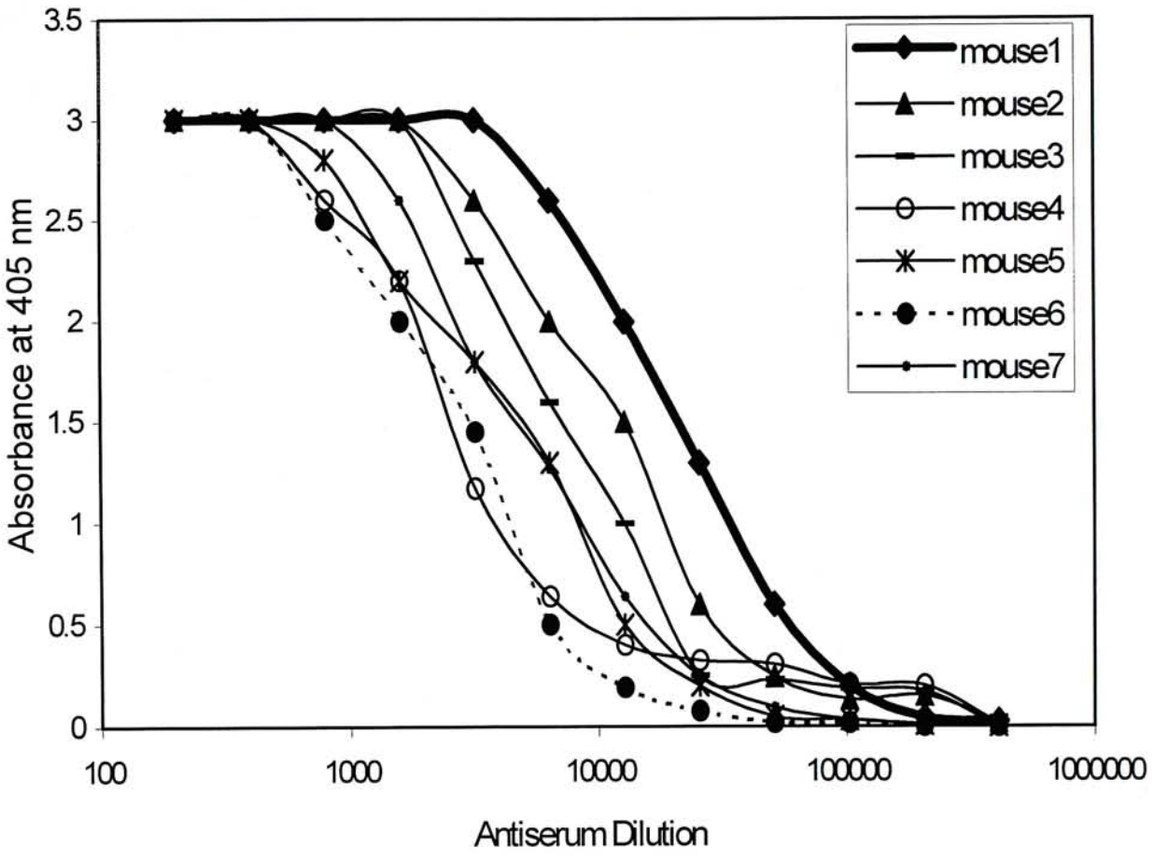


Figure 3.4 Anti-apoB100 antibody (IgG) activity in the sera taken on day 4 after the 3rd booster of apoB100 injection in seven mice. Anti-apoB100 activity was measured by ELISA.

this stage, E8 was probably a homogeneous clone.

In order to associate the specificities of these monoclonal antibodies against different apolipoproteins, clone B6 is named AB6 from hereon to reflect its specificity for apoA-I and the clone E8 is named BE8 to reflect its specificity for apoB100. By isotyping, both the AB6 and BE8 have IgG_1 type of heavy chain and the κ type of light chain.

3.3.2 Purification of monoclonal antibody from ascites

Elution profiles for purification of AB6 and BE8 by protein A-Sepharose CL-4B affinity chromatography are shown in Figure 3.5 and 3.6. In both cases, the antibody was eluted in a sharp peak. Figure 3.7 illustrates the purity of AB6 and BE8. The purity achieved was almost 100%. By 12.5% SDS-PAGE, the antibodies were resolved into two bands. The band with an apparent molecular weight of 55 kDa was the heavy chain while the band with an molecular weight of 25 kDa was the light chain of IgG.

3.3.3 Western blotting analysis of AB6 and BE8

The specificities of AB6 and BE8 were confirmed by western blotting. As shown in Figure 3.8, AB6 reacted with a single band in human serum with a molecular weight of 28 kDa (Figure 3.8b). AB6 bound only to apoA-I but with no activity to other serum proteins. BE8 bound only to apoB100 but not to other LDL proteins (Figure 3.9). BE8 also showed no reactivity with other serum proteins in immunoblotting with human serum which was separated by 12.5% SDS-PAGE (LDL could not enter the gel) (data not shown).

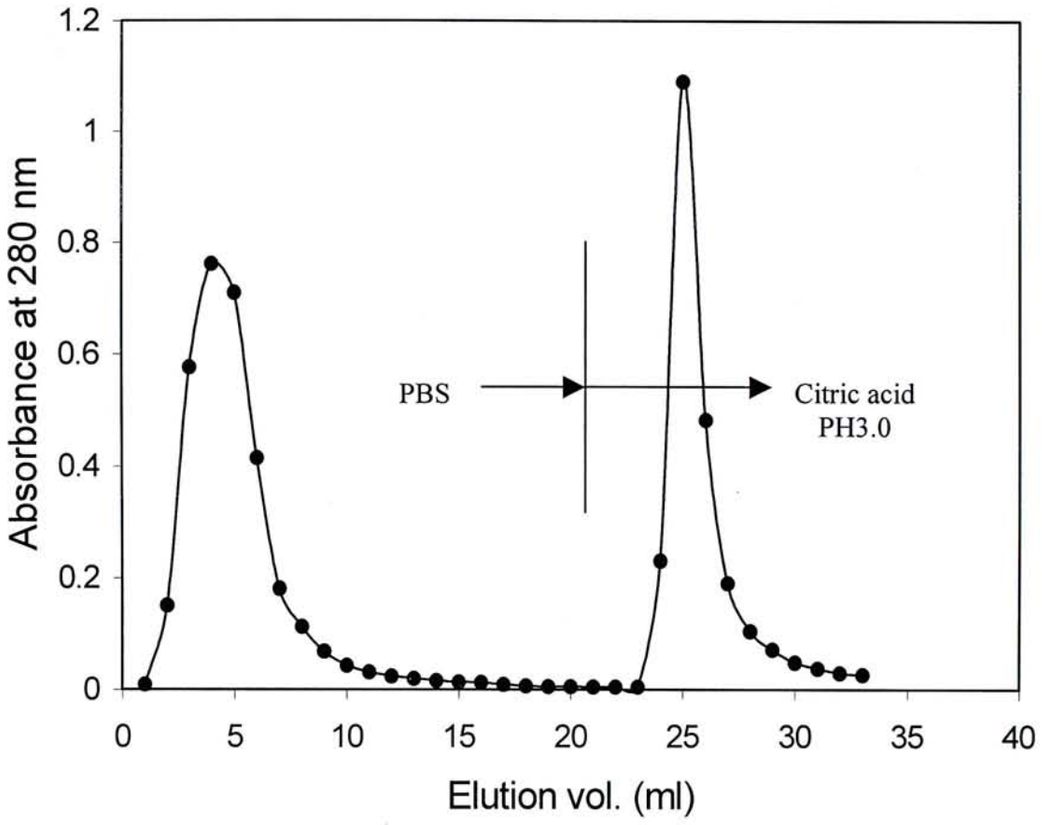


Figure 3.5 Protein G-Sepharose CL-4B chromatography of purification of monoclonal antibody AB6. After loading with sample, the column was washed with 20 column volumes of PBS at a flow rate of 1 ml/min. Subsequently, the column was eluted with 0.1 M citric acid (pH 3.0).

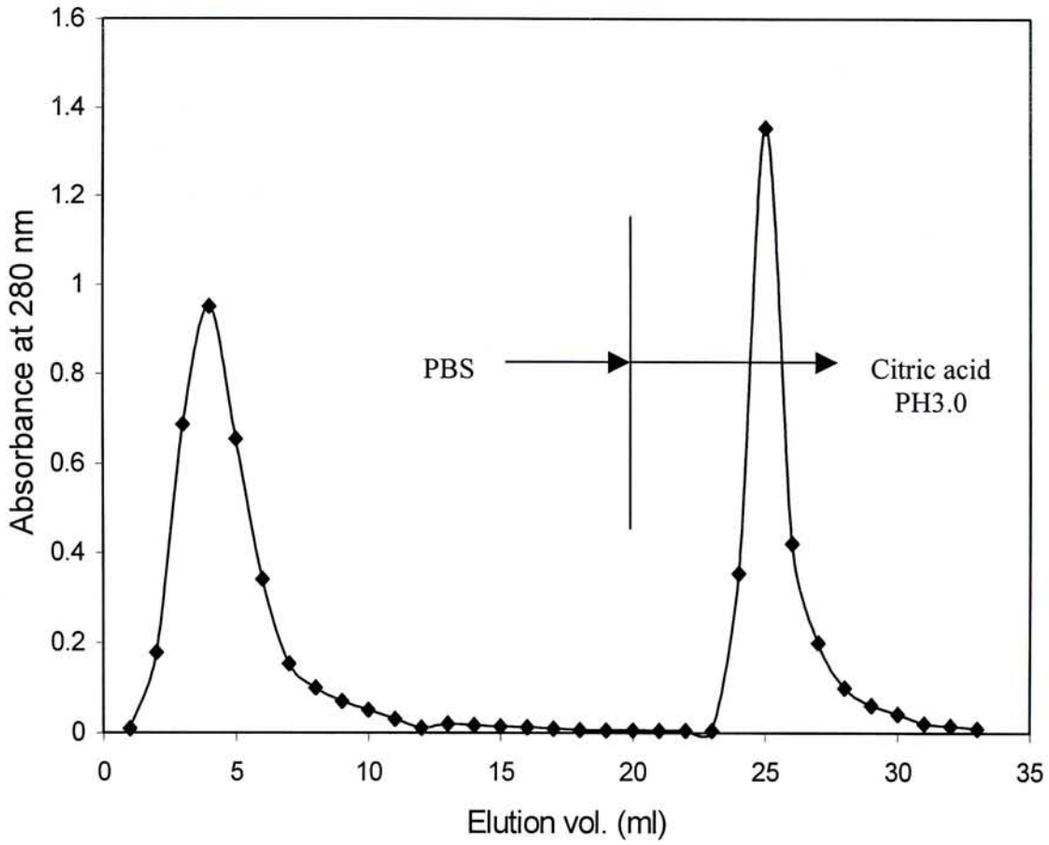
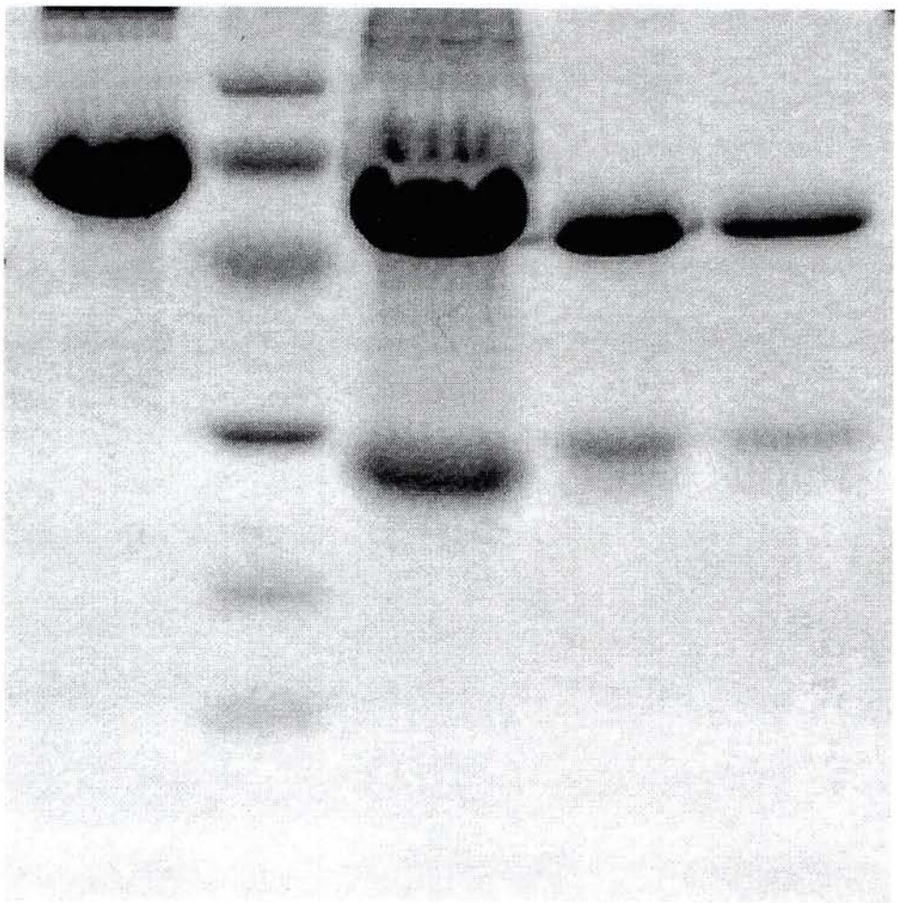


Figure 3.6 Protein G-Sepharose CL-4B chromatography of purification of monoclonal antibody BE8. The procedure was the same as the AB6 purification.



A B C D E

Figure 3.7 SDS-Polyacrylamide (12.5%, reducing) gel electrophoresis of monoclonal antibodies. (A) standard: albumin, 67 kDa; (B) low molecular weight marker; (C) protein precipitate from 45% ammonium sulfate precipitation of ascites; (D) purified AB6; (E) purified BE8.

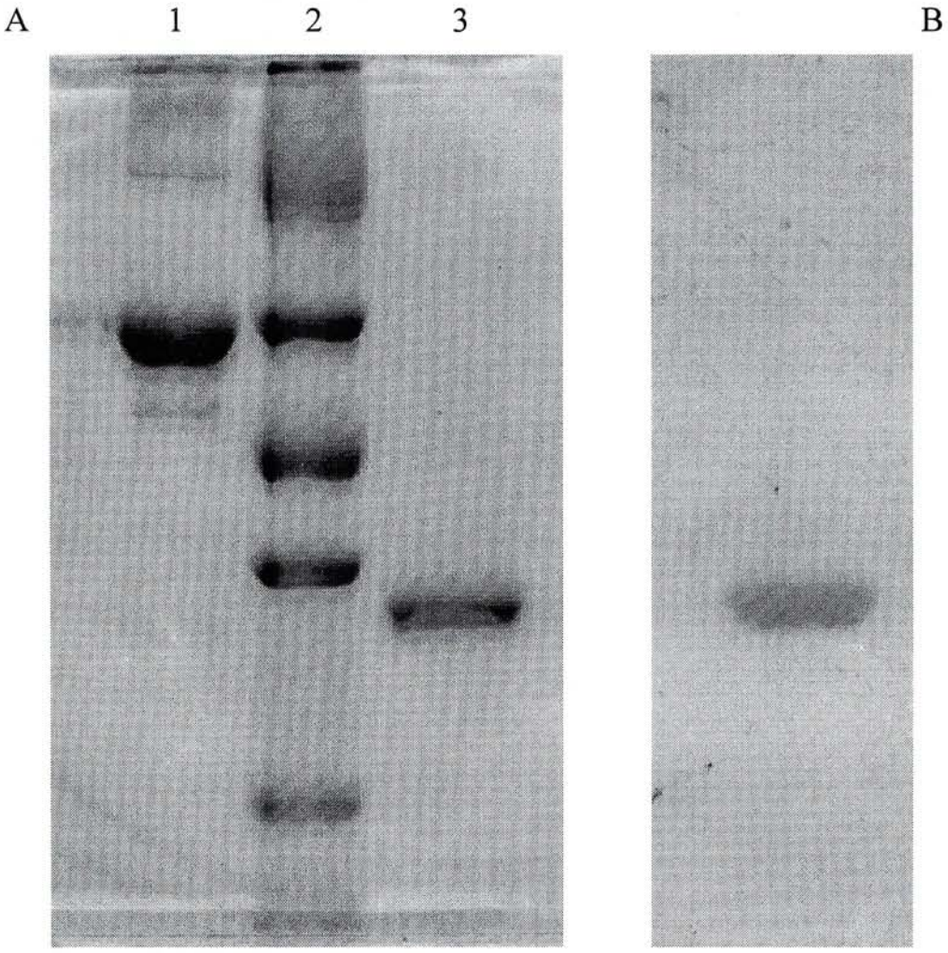


Figure 3.8 Analysis of the specificity of AB6 by SDS-PAGE and immunoblotting. (A) Various proteins were separated on 12.5% SDS-PAGE: 1. Albumin; 2. Low molecular weight marker; 3. ApoA-I. (B) Human serum proteins were separated on 12.5% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with AB6 and reactive bands were detected with ALP-conjugated secondary antibody.

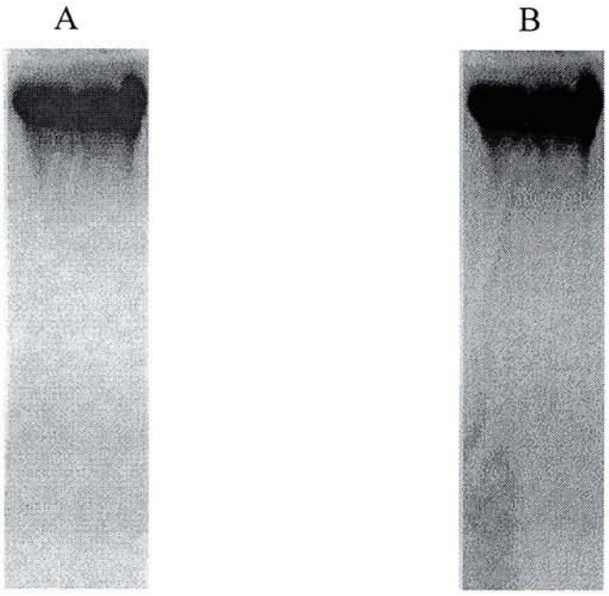


Figure 3.9 Analysis of the specificity of BE8 by SDS-PAGE and immunoblotting. (A) 5% SDS-PAGE of LDL. (B) The proteins were blotted onto nitrocelluose membrane and incubated with BE8. The reactive band was detected with ALP-conjugated secondary antibody

3.3.4 Monoclonal antibody titration curve for apolipoproteins by radioimmunoassays

The binding of ^{125}I-labeled apoA-I at different concentrations of monoclonal antibody AB6 is shown in Figure 3.10. At low concentrations of AB6, i.e., 200 ng/ml to 7 μg/ml, a linear increase in the binding of ^{125}I-labeled apoA-I was observed with increasing concentrations of AB6. The maximum binding was obtained at 62.5 μg/ml of antibody; however, at higher concentration, a decreased in apoA-I binding was observed and this probably indicates a prozone phenomenon. Thus, when the concentration of AB6 was increased to 62.5 μg/ml, all epitopes on apoA-I were saturated and the addition of more AB6 increased the steric interference and reduced the interaction between the antibody and apoA-I. 50% of the maximum binding was observed when the AB6 concentration was 2 μg/ml.

Figure 3.11 is the binding curve of monoclonal BE8 for ^{125}I-labeled apoB100. A linear range was obtained from 2 to 30 μg/ml. Maximum binding was observed at 300 μg/ml and 50% of the maximum binding was determined to be at 7μg/ml of BE8.

3.3.5 Competitive displacement analysis of AB6 and BE8

Addition studies to characterize the specificities of AB6 and BE8 were carried out by competitive displacement analysis. From the results shown in Figure 3.12, the addition of LDL, VLDL, BSA or lipoprotein-depleted serum (LPDS) at concentrations of 64 ng/ml to 1 mg/ml did not displaced bound ^{125}I-labeled apoA-I from AB6. On the other hand, apoA-I, HDL and whole serum demonstrated a concentration-dependent parallel displacement of apoA-I. The IC_{50} for apoA-I was 8 μg/ml, for HDL was 50 μg/ml and for whole serum was 70 μg/ml. These results suggest no cross-reactivity of AB6 with LDL, VLDL, BSA or other serum proteins.

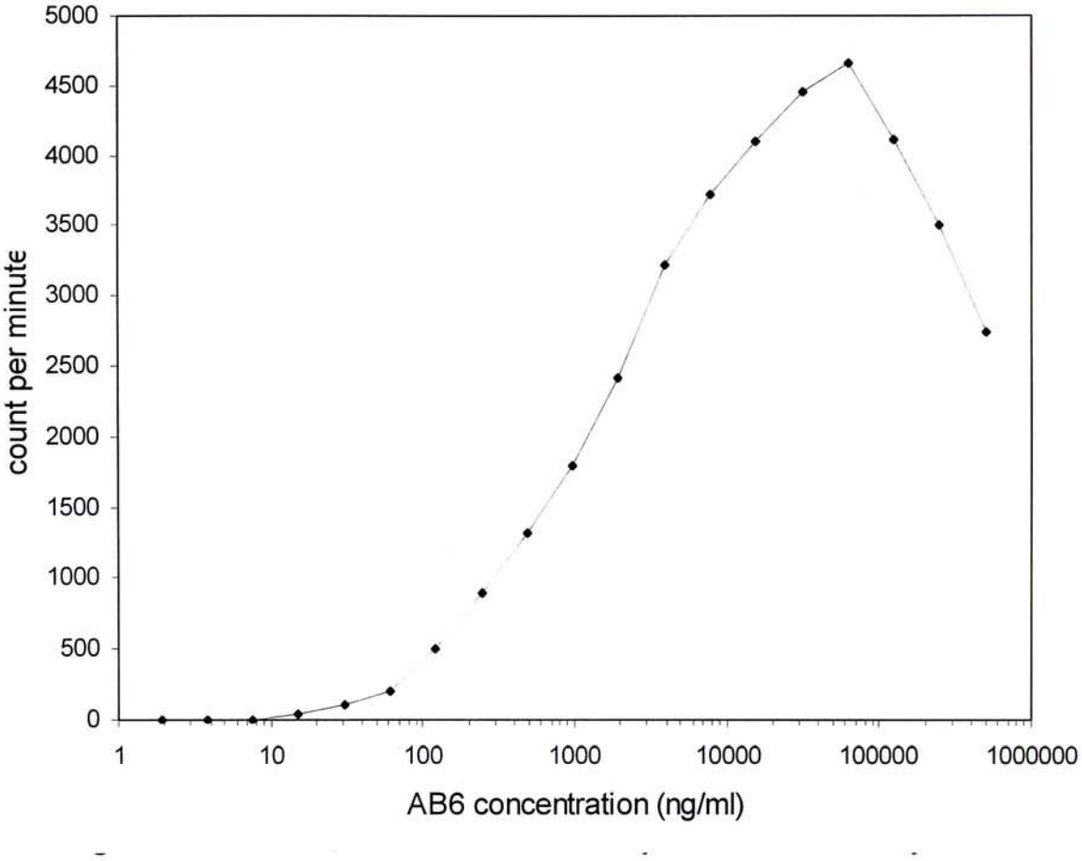


Figure 3.10 Titration curve of AB6 by radioimmunoassay.

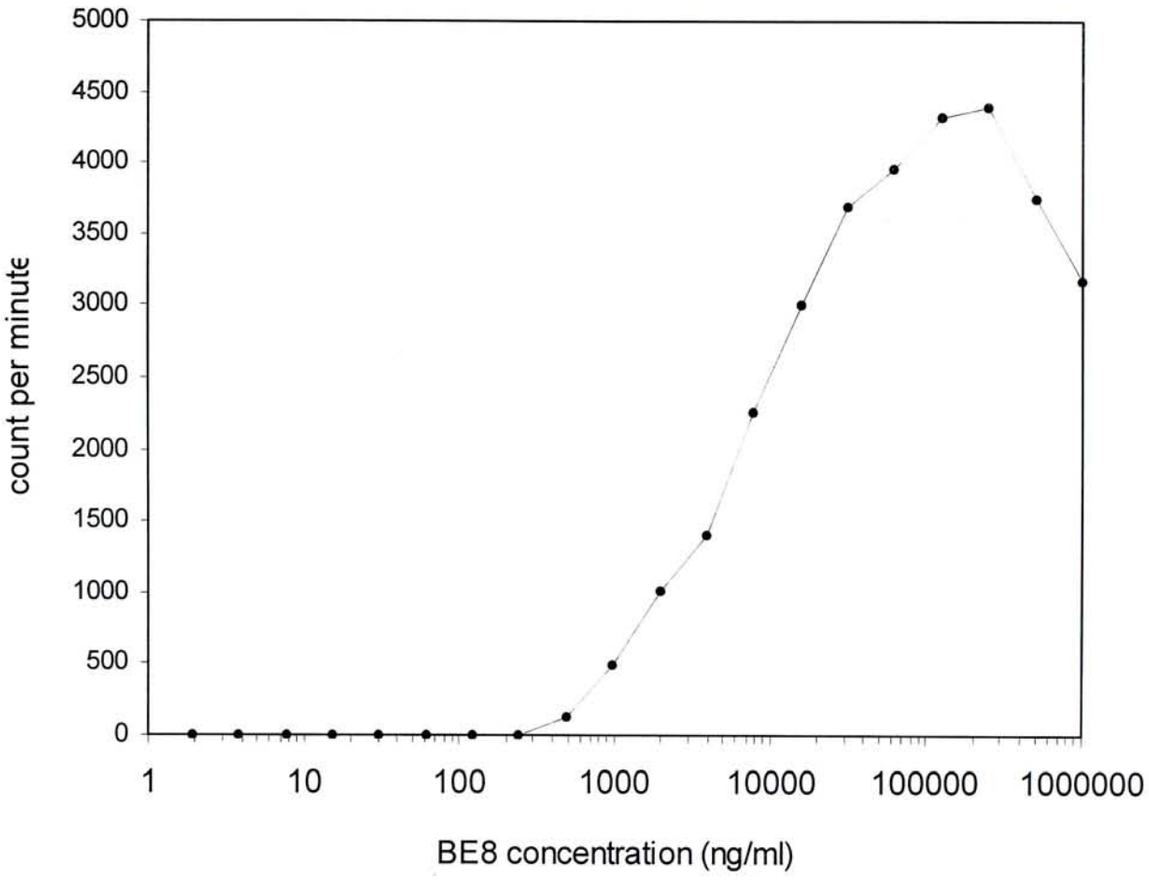


Figure 3.11 Titration curve of BE8 by radioimmunoassay.

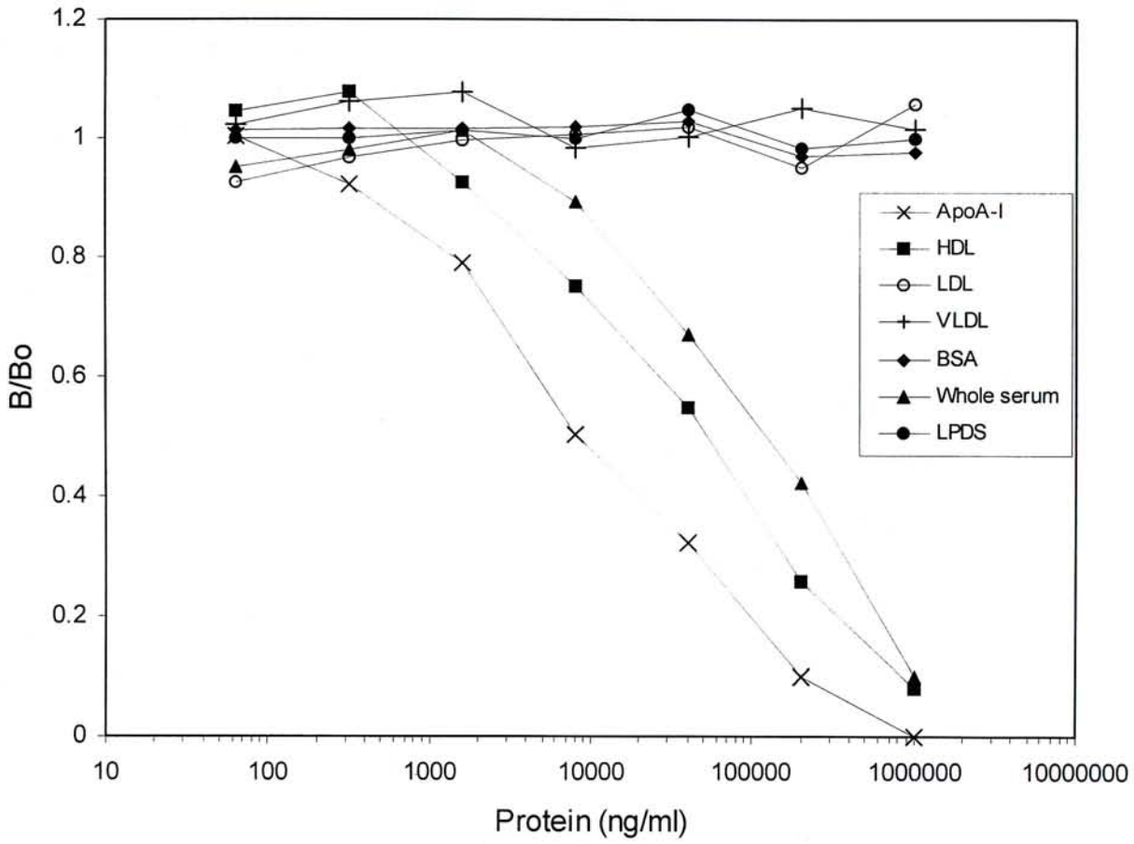


Figure 3.12 Competitive displacement analysis of AB6.Iodinated apoA-I was co-incubated with AB6 and various total protein concentrations of purified apoA-I, HDL, LDL, VLDL, human whole serum, BSA, and lipoprotein-depleted serum (LPDS). The immunocomplex was precipitated by Sac-cel and its radioactivity was measured by gamma-counter.

As depicted in Figure 3.13, the addition of HDL, BSA or LPDS at concentrations of 64 ng/ml to 1 mg/ml did not displaced BE8 bound ^{125}I-labeled apoB100 from BE8. A concentration-dependent parallel displacement of ^{125}I-labeled apoB100 was observed for apoB100, LDL, VLDL and whole serum. The IC_{50} for apoB100 was 35 μg/ml, LDL was 60 μg/ml, VLDL was 70 μg/ml and whole serum was 100 μg/ml. This suggests that no cross-reactivity of BE8 against HDL, BSA or other serum proteins.

3.4 Discussion

Two hybridoma fusions were performed for the production of monoclonal antibodies against apoA-I and apoB100. In these two fusions, the efficiencies were 23.2% and 23.7%. Among the successfully fused cells, 3.4% (3 out of 89) and 5.5% (5 out of 91) were hybridomas secreting antibodies against apoA-I and apoB100, respectively. Various fusion efficiencies have been reported in different studies ranging from 10% (Berghman et al. 1988) to 90% (Chen et al. 1996). Many parameters can influence the fusion efficiency, and the most crucial one is the fusion method. Electrofusion (Hewish and Werkmeister 1989) and laser fusion (Wiegand et al. 1987) were introduced in the last decade and these methods can significantly increase the fusion efficiency. The biotin-avidin method of linking B cells with myeloma cells has also been employed to increase fusion efficiency (Wojchowksi and Sytkowski 1986).

From the five apoA-I and three apoB100 positive clones, we were only successful in obtaining one clone from each case. The lost of positive clone during cloning is not uncommon. The fused cells which carry an initial double load of chromosomes tend to take longer to replicate. These cells can lose chromosomes

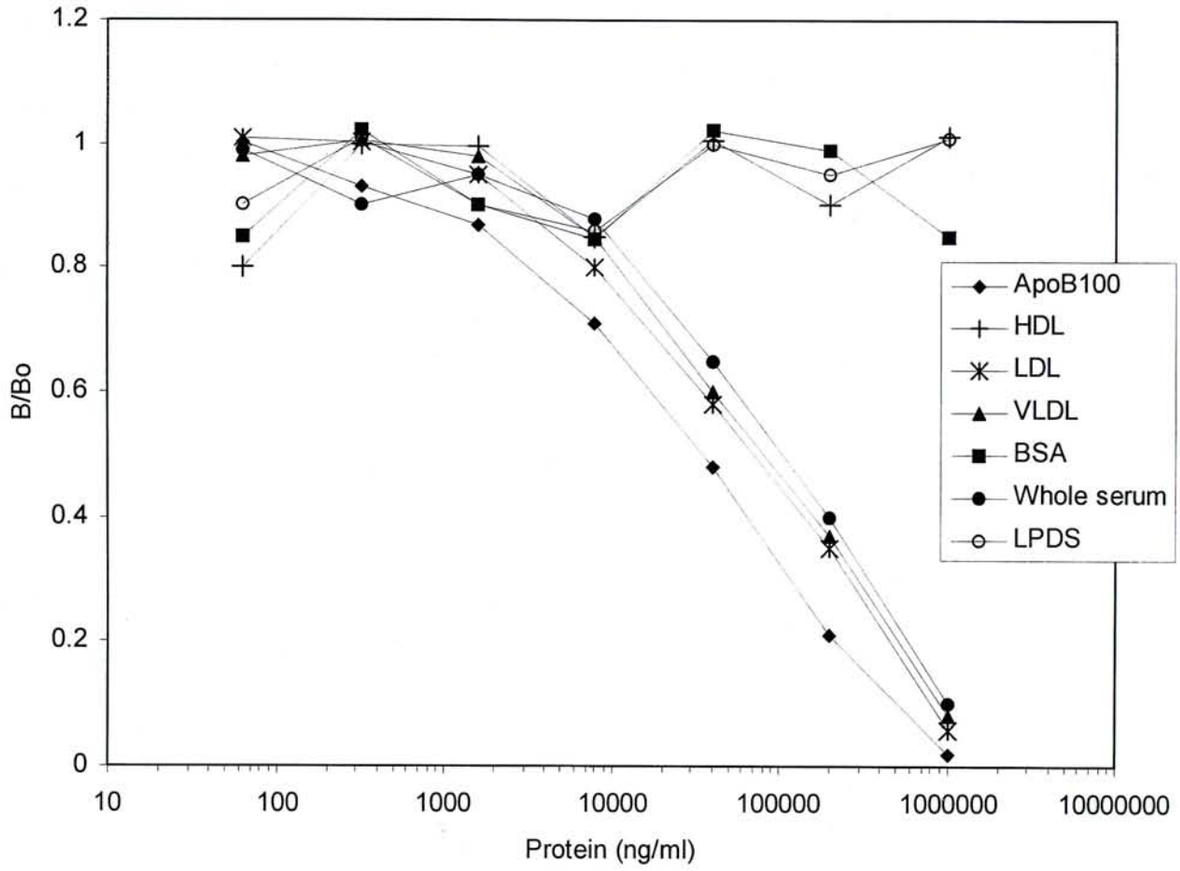


Figure 3.13 Competitive displacement analysis of BE8.Iodinated apoB100 was co-incubated with BE8 and various total protein concentrations of HDL, LDL, VLDL, human whole serum, BSA, and lipoprotein-depleted serum (LPDS). The immunocomplex was precipitated by Sac-cel and its radioactivity was measured by gamma-counter.

very fast and if these chromosomes include mouse chromosome 12, which contains the immunoglobulin heavy chain gene, or chromosome 6 which contains the κ light chain gene, the ability of the fused cells to secrete the appropriate antibody will be lost. The second possible explanation is that the screening assay produces false positive signal. The unfused B cells which secrete specific antibody can grow in selecting medium for a few days, and the positive signal obtained in the initial screening may from these unfused B cells. The myeloma cell line used in this study may also contribute to the lost of positive hybridomas. NS-1 can synthesize κ light chain. Although NS-1 cannot secrete free κ light chain, it can however, incorporate it into a heavy chain and secrets it if the NS-1 is fused with a B cell (Köhler and Milstein 1976). In this case, the hybrid antibody molecule will have low or no binding affinity for the antigen because it lacks a binding site. The most common non-secretor myeloma cell lines are listed in Table 3.3.

Two monoclonal antibodies, AB6 and BE8, against apoA-I and apoB100, were successfully produced and purified in this study. Their relative affinities to their respective antigens were determined from their titration curves (Figure 3.10 and 3.11). For a constant amount of labeled antigen in the reaction, less amount of high affinity antibody is required to bind 50% of the antigen than is required by a low affinity antibody. Using this a criterion, we can conclude that AB6 has a higher affinity to apoA-I than BE8 to apoB100.

The specificities of AB6 and BE8 were demonstrated by western blotting and competitive displacement analysis. Both of these studies showed that AB6 is specific to apoA-I and apoA-I-containing lipoprotein while BE8 is specific to apoB100 and apoB100-containing lipoproteins. The two monoclonal antibodies show no cross-reactivity with other lipoproteins or serum proteins.

Table 3.3 Commonly used myeloma cell lines in hybridoma production

Cell line	Derived from	Fusion efficiency	Hybridoma stability
κ light chain secretor			
P3/NS1-Ag4-1 (NS-1)	X63	+++	+++
Non-secretor			
P3-X63-Ag8-653	X63 – Ag8	++	+++
Sp2/0-Ag14 (Sp2)	X63 – Ag8 x BALBc	++	+++
F0	Clone of Sp2	+++	+++
NS0/1	NS1 – Ag4-1	++	+++

Apolipoprotein is embedded in the lipid layer of the lipoprotein and it is possible to produce a monoclonal antibody with its recognition site shielded by the lipid layer. The parallel displacement curves observed for purified apolipoproteins, intact lipoproteins and whole serum suggest that AB6 and BE8 recognize these entities identically. So it is likely that the recognition site of AB6 and BE8 are located on the surface of lipoprotein rather than those epitopes internally shielded by lipid layers.

Many studies have been focused on the conformational states of apolipoproteins in lipid-free and lipid-bound states (Jonas 1986; Wetterau and Jonas 1982). These studies suggest that the conformation of the apolipoproteins are different in these two states, and there may be more than one conformation for lipid-bound apolipoprotein (Jonas et al. 1977; Wetterau and Jonas 1983). The production of a monoclonal antibody which can recognize all these conformations with same affinity will refine the development of an immunoassay for detecting lipoproteins in their natural serum environment. Along this lining reasoning, it is better to use an antibody for immunoassay which recognizes free and lipid-bound apoA-I with same affinity. This is true, because there are free apoA-I molecules in the serum derived from HDL catabolism (Liang et al. 1994; Kunitake et al. 1985).

In summary, we have produced two hybridoma cell lines for anti-apoA-I and anti-apoB100 monoclonal antibodies. These antibodies are only specific to their target antigens with no cross-reactivity to other lipoproteins or serum proteins. Their recognition sites are probably an apolipoprotein fragment exposed on the surface of the lipoproteins as they have same binding affinity to free apolipoprotein and whole lipoprotein.

Chapter 4

Enzyme-linked immunosorbent assay (ELISA) for Apolipoprotein A-I

4.1 Introduction

Enzyme is one of the most versatile labeling substances in immunoassays. The term ELISA (enzyme-linked immunosorbent assay) was introduced in 1971 (Engvall and Perlmann 1971). The first ELISA for determination of apoA-I was developed in 1983 (Koritnik and Rudel 1983). More recently, several types of ELISA, either direct or competitive, have been reported for apoA-I measurement (Bury and Rosseneu 1988; Bury and Rosseneu 1985; Dubois et al. 1987; Barkia et al. 1988; Betard et al. 1987). ELISA is well suited to apolipoproteins measurement because it requires small amount of antibody. It does not require the use of radioisotopes and can be easily automated. Finally, its accuracy and reproducibility compare well with other assays.

4.1.1 Alkaline phosphatase (ALP)

ALP (orthophosphoric-monoester phosphorylase, alkaline optimum, E.C. 3.1.3.1) is one of the most commonly used enzymes in ELISA. It represents a large family of isoenzymes. ALP used in ELISA generally comes from bovine intestinal mucosa or *E. coli*. Bovine intestinal ALP has a molecular weight of about 140,000. The active site contains two zinc atoms and one magnesium atom, both of them are essential for activity (Kim and Wyckoff 1991). The exact biological function of ALP is still uncertain. ALP isozymes can cleave phosphomonoester groups from a wide variety of substrates. Typical phosphate acceptor additives include diethanolamine,

Tris, and 2-amino-2-methyl-1-propanol. The presence of these additives in substrate buffers can dramatically increase the sensitivity of the ELISA.

ALP is commonly used in ELISA due to its stability. It can withstand moderately high temperature and can maintain enzyme activity over extended periods of substrate development.

4.1.2 Conjugation methods

In a typical sandwich ELISA, antibody is conjugated with an enzyme for the detection of the solid phase-bound analyte. In a conjugation reaction, both the antibody and enzyme are chemically modified. Functional groups of the two molecules are covalently linked together by a bifunctional cross-linker. Cross-linker can be classified on the basis of their length, homobifunctional or heterobifunctional, and chemically or photochemically reactive. In this study, a heterobifunctional cross-linker N-Succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) was used to link up the anti-apoA-I antibody with the alkaline phosphatase enzyme.

SMCC contains an amine-reactive N-hydroxysuccinimide (NHS) ester on one end and a sulfhydryl-reactive maleimide group on the other. SMCC possesses the most stable maleimide functional group and allows activation of either enzyme or antibody via the amine-reactive NHS ester end. The maleimide-activated intermediate can be purified away from excess cross-linker and reacts with the second protein (Figure 4.1). The two-step nature of this process limits polymerization of the conjugated proteins and provides control over the extent and sites of cross-linking.

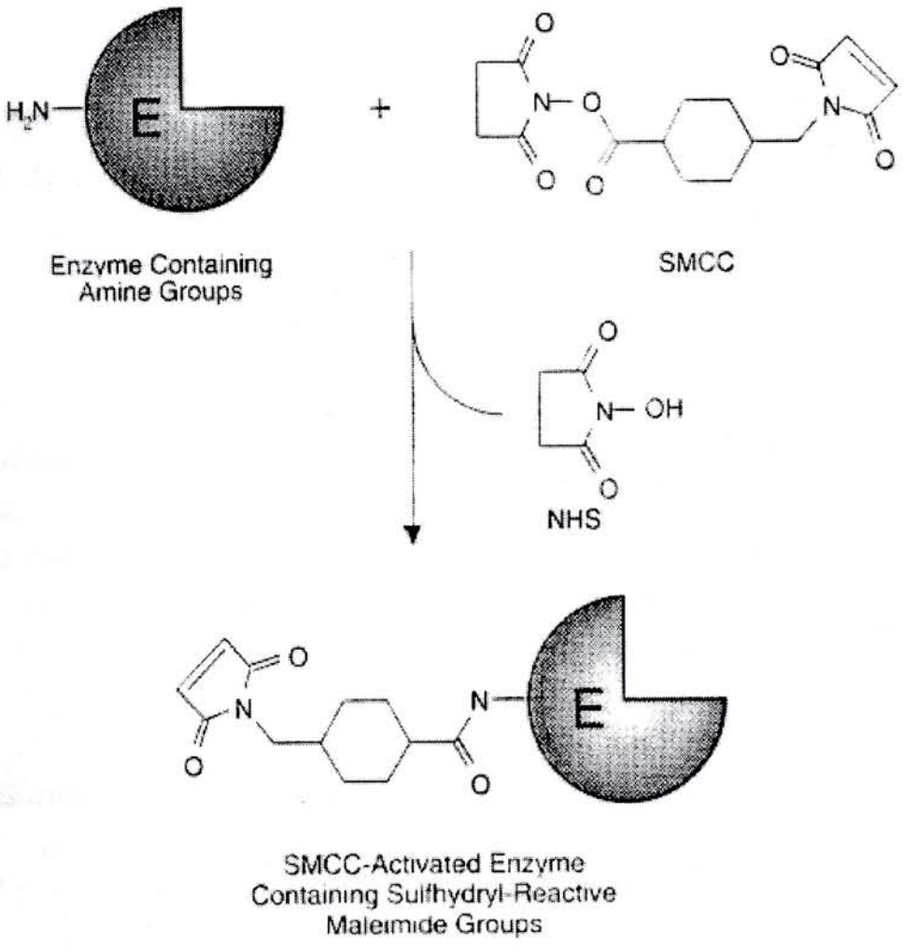


Figure 4.1 The reaction of SMCC with the amine group on enzyme molecule yields a maleimide-activated derivative capable of coupling with sulfhydryl-containing antibody molecules.

In this study, the alkaline phosphatase was activated by SMCC and subsequently conjugated with the monoclonal antibody. Since antibodies typically do not contain free sulfhydryls accessible for conjugation, 2-mercaptoethylamine (MEA) can be used to cleave the immunoglobulin molecule into two half each possessing one antigen binding site and the requisite sulfhydryls (Figure 4.2).

4.1.3 Design of the immunoassay format

In this study, a noncompetitive two-site (sandwich type) immunoassay will be developed for the measurement of apoA-I in which a combination of monoclonal and polyclonal antibodies is used (Figure 4.3). Polyclonal antibody is preferred to be the capturing antibody because they can capture the analyte with more than one epitope. The rate of dissociation of any one antibody-epitope binding is the same as for a simple interaction, but because the antigen is still held by other interactions, the overall rate of dissociation is very slow. Monoclonal antibody will be used as the detecting antibody because it can offer better specificity than polyclonal antibody. Because sandwich immunoassays involve two antibodies, they are more specific than competitive assays. Cross-reacting substances which interfere with the competitive assays give no signal in sandwich assays, because it is unlikely that they will bind to both the capture and the detection antibodies.

4.1.4 Modified solid-phase: Protein A antibody-capture ELISA (PACE)

The antibody binding capacity of a polystyrene microtiter plate can be increased by coating the wells with protein A or protein G prior to the addition of an antibody. The signal strength and sensitivity of many types of ELISA are dependent on the amount of antibody available to capture the antigen. When microtiter plates

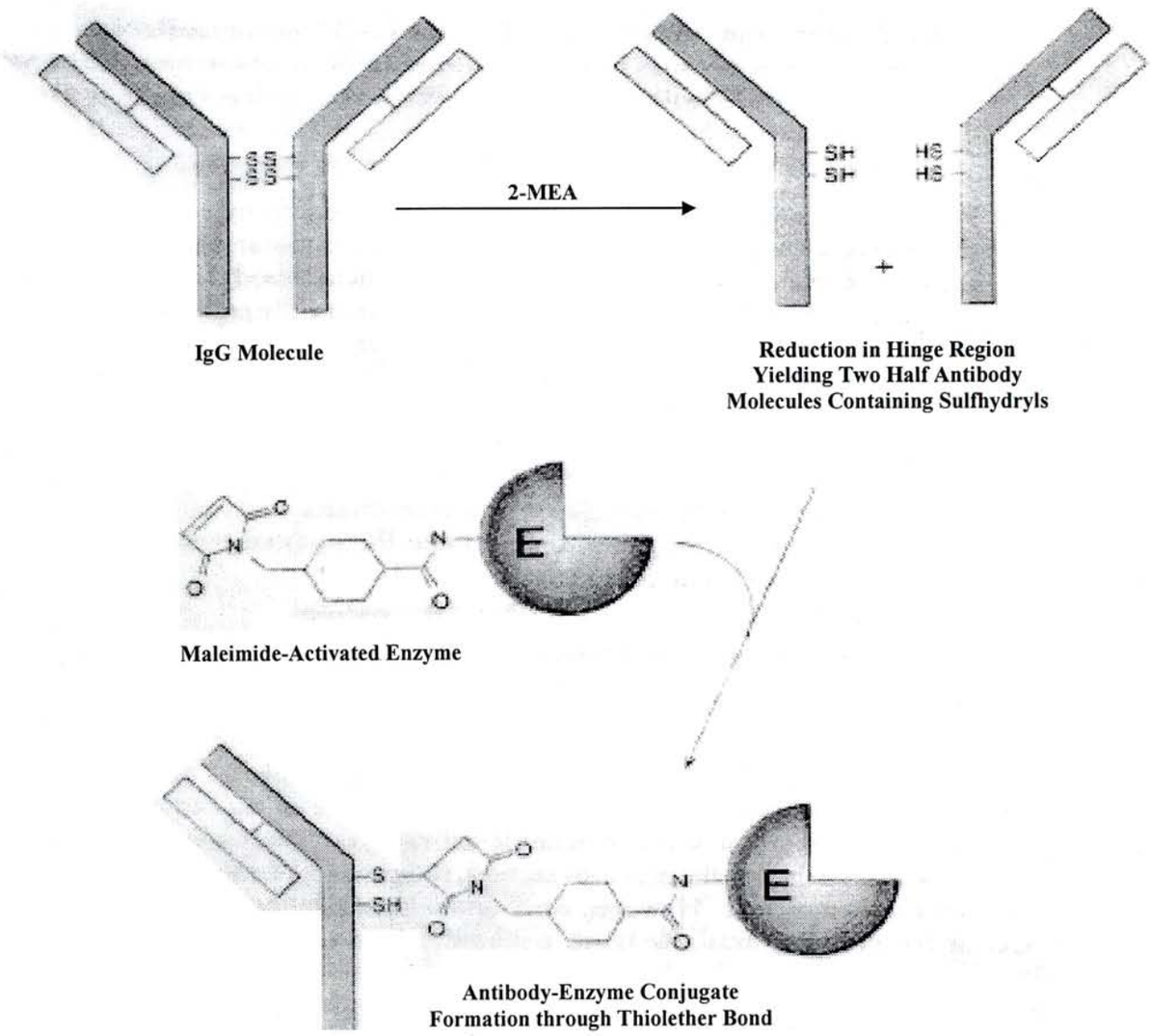


Figure 4.2 Reduction of the disulfide bonds within the hinge region of an IgG molecule produces half-antibody molecules containing thiol groups. Reaction of these reduced antibodies with a maleimide-activated enzyme creates a conjugate through thioether bond formation.

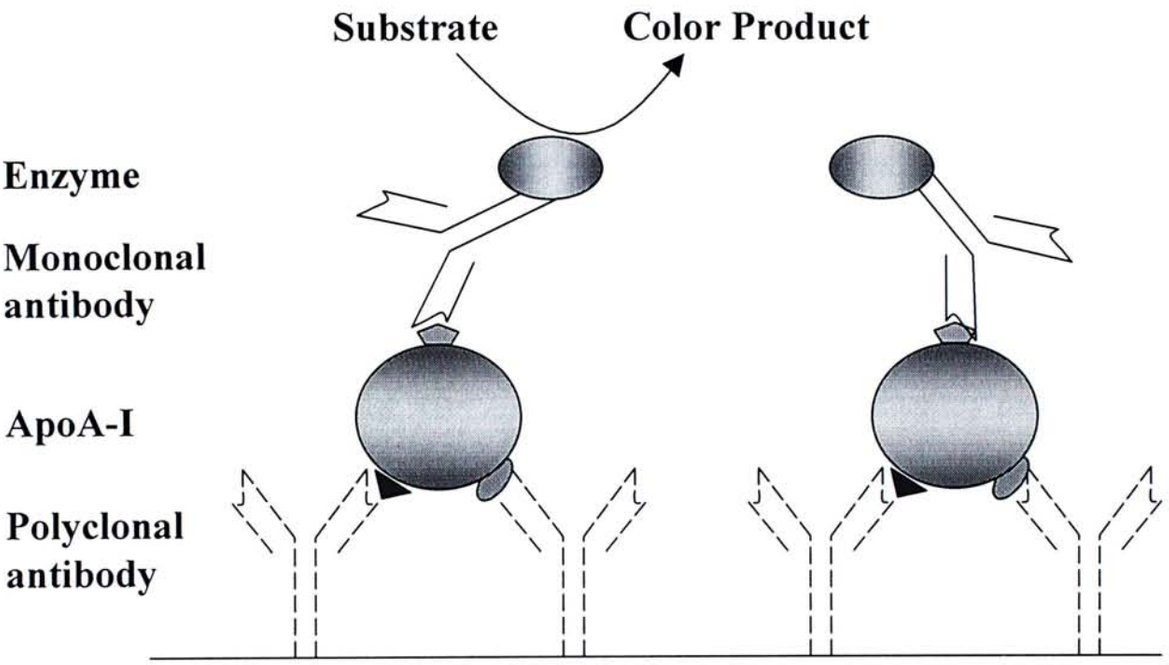


Figure 4.3 Sandwich ELISA using a combination of polyclonal and monoclonal antibodies.

are pre-coated with proteins A or G, the amount of antibody bound per well can be significantly increased leading to the capture of more antigen (Ngai et al. 1993). In this study, a new ELISA format, PACE (protein A antibody-capture ELISA), is developed for the quantification of apoA-I.

4.2 Methods

4.2.1 Conjugation of AB6 with maleimide activated alkaline phosphatase

Monoclonal antibody AB6 at a concentration of 1mg/ml was dissolved in conjugation buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH7.2, containing 10 mM EDTA). 6 mg of 2-mercaptoethylamine (MEA) was added to the antibody and incubated for 90 minutes at 37°C for generating thiol groups in the antibody molecules. The reduced AB6 and excess MEA were separated by passing through a desalting column. The effluent was collected in 0.5 ml fractions and the protein content of each fraction was monitored at 280 nm. The fractions contained antibody were pooled together. The antibodies were then mixed with maleimide-activated alkaline phosphatase (purchased from Pierce Chemical Company) to obtain the desired molar ratio of antibody-to-enzyme (1:4). This ratio was previously determined to yield 0.5 to 2 moles of ALPs per mole of IgG. The conjugation reaction was performed at room temperature for one hour.

4.2.2 Titration curve of AB6-ALP conjugate

Microtiter plates were coated separately with HDL, LDL, VLDL, LPDS, or BSA (200 μl per well with a protein concentration of 25 μg/ml). After washing and blocking as previously described, 100 μl of AB6-ALP at various dilutions were added. The plates were then incubated at 37°C for two hours. After washing three

times with PBSMT and two times with PBST, 100 μl substrate solution was added to assess the amount of enzyme-antibody conjugate bound.

4.2.3 Calibration curve of apoA-I sandwich ELISA

Microtiter plates were coated for 16 hours at 4°C with 200 μl of PBS containing 2 μg purified IgG from rabbit anti-apoA-I serum in each well. The plates were washed three times with PBSMT and blocked by 1% BSA in PBS. The plates were washed three times with PBST to remove excess BSA. Plates prepared in this manner could be used for up to one month if stored in a humidified chamber. To create the calibration curve, 200 μl standard apoA-I at concentrations ranging from 10 μg/ml to 9.8 ng/ml was added and incubated at 37°C for an hour. PBS was used for dilution. After the plates were washed three times with PBSMT, 100 μl of AB6-ALP conjugate (100 fold dilution from stock) was added and incubated at 37°C for an hour. The unbound conjugate was washed out three times with PBSMT and then two times with PBST. 100 μl freshly prepared substrate solution pNpp was then added for color development. The reaction was stopped by adding 50 μl of 0.4 M NaOH. Absorbance of the individual wells was determined at 405 nm using a plate reader.

4.2.4 Measurement of apoA-I by Protein A antibody-capture ELISA

The procedures used in the protein A antibody-capture ELISA was the same as the sandwich ELISA except the microtiter plates were pre-coated with Protein A before the coating of the capturing antibody,. The protocol of Protein A pre-coating is as follow: 50 μl of Protein A at a concentration of 20 μg/ml was added to each well. The microtiter plates were then incubated at 37°C for an hour and unabsorbed Protein A was washed off by PBST three times. Uncoated sites were blocked by

incubating with 100 µl 1% BSA in PBS at 37°C for one hour. After washing three times with PBST, the plates were ready for coating of the capturing antibody as described in section 4.2.2.8.

4.3 Results

4.3.1 Characterization of AB6-ALP conjugate

In order to determine the working dilution of the antibody-enzyme conjugate, a binding curve of AB6-ALP to HDL immobilized on microtiter wells was determined (Figure 4.4). At 40-fold dilution (with a protein concentration of 101.7 µg/ml) or below, an absorbance of 0.7 was obtained. Beyond 40-fold dilution, activity gradually decreased with increasing dilution. When the antibody-ALP conjugate was diluted to 2000-fold, absorbance decreased to 0.1. In order to economize on the use of the antibody-ALP conjugate for our subsequent assay, a dilution was selected so that it will give a reasonable level of sensitivity without using excess amount of the reagent. Base on this criterion, the working dilution of the conjugate was established to be around 100-fold. Moreover, as shown in Figure 4.4, the conjugate had no cross-reactivity with other lipoproteins and therefore conjugating AB6 with ALP did not seem to have any effects on the specificity of the antibody.

4.3.2 Calibration curve for the measurement of apoA-I

Figure 4.5 describes the standard curves for apoA-I determination using sandwich ELISA and protein A antibody-capture ELISA (PACE). Both assays were able to detect apoA-I level in the range of 0.2 to 1.2 µg/ml. Compared with the conventional sandwich ELISA, PACE showed an average increase in signal strength of 260%.

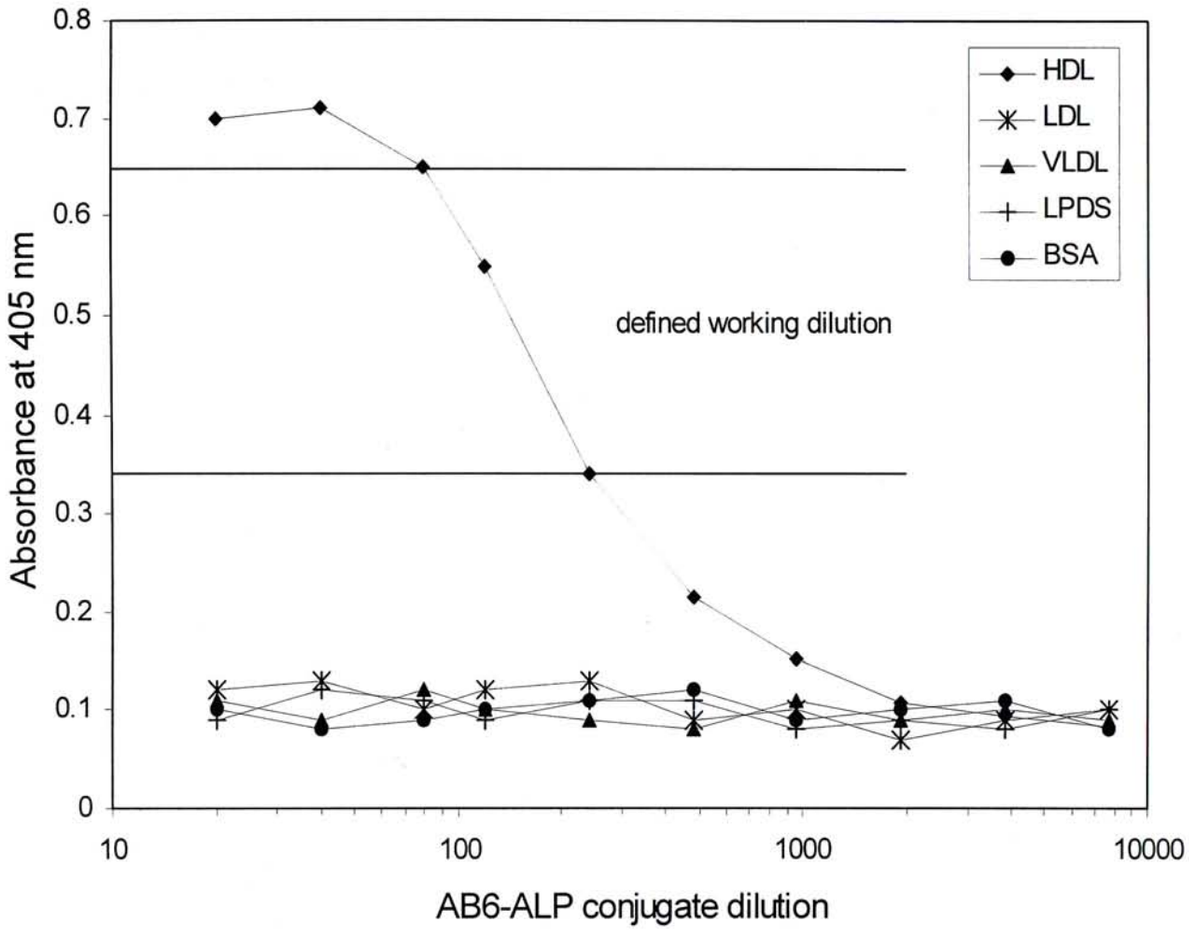


Figure 4.4 Titration curve of AB6-ALP conjugate. Specificity was tested in wells coated with HDL, LDL, VLDL, and LPDS, all at 25 μg/ml, and background with BSA-coated wells. The working dilution of the conjugate was set in the linear part of the dilution curve. Absorbance at 405 nm was measured after 30 minute color development.

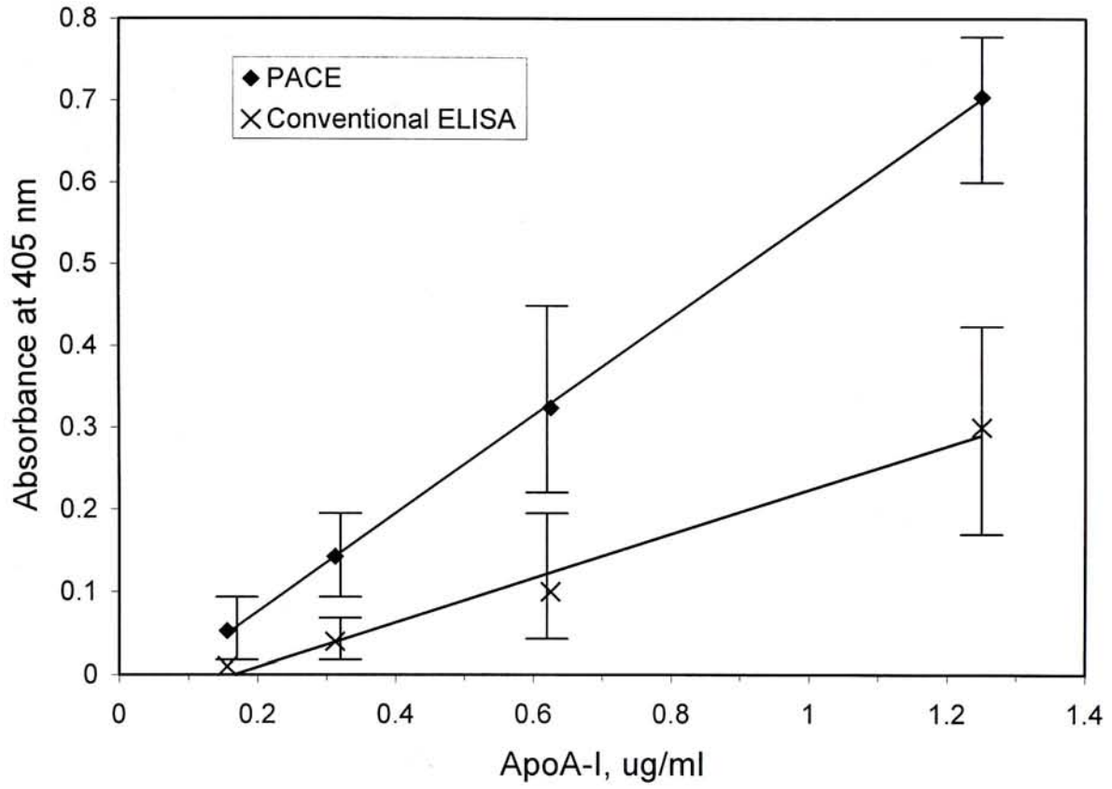


Figure 4.5 Standard curves of the conventional sandwich ELISA and Protein A antibody-capture ELISA for the measurement of apoA-I. Each assay was repeated twice.

The inter- and intra-assay variabilities of the PACE method were determined by assaying seven samples, with apoA-I concentrations from 0.4 to 1.0 μg/ml, 6 times on the same day, and on five separate days. As summarized in Table 4.1, the mean intra- and inter-assay CVs for the entire assay range were 5.96 and 7.83%, respectively.

4.4 Discussion

Because of its stability and high sensitivity, alkaline phosphatase was chosen in this study to prepare the antibody-enzyme conjugate. The AB6-ALP conjugate had a working range between 80- to 120-fold dilution when tested against HDL. Compared with commercial preparations of enzyme conjugates, our conjugation efficiency was relative low. To increase the efficiency, the reaction time and temperature can be prolonged or increased. Moreover, the signal can also be amplified by increasing the ratio of enzyme to antibody. However, too many enzymes may have an adverse influence on the binding of the antibody to the antigen.

In this study, we have used a polyclonal antibody preparation and a monoclonal antibody-enzyme conjugate to develop a sandwich ELISA for apoA-I measurement. The signal strength of this assay was found to be low and it was probably due to either the low binding capacity of the polyclonal antibody onto the polystyrene microtiter plates or the loss of affinity to the antigen after the antibody was adsorbed onto the plastic surface. If these problems were real, protein-A antibody capture ELISA may provide a solution for them. Past report showed that the wells pre-coated with Protein A could absorb more antibody (Ngai et al. 1993). Furthermore, since only the Fc fragment binds to protein A, immobilization of the capturing antibody in the Fc region can minimize the conformation change of the

Table 4.1 Precision of Protein A antibody-capture ELISA (PACE) for ApoA-I

ApoA-I concentration, μg/ml	CV, %	
	Intra-assay	Inter-assay
0.4	5.37	7.94
0.5	6.21	8.12
0.6	6.12	7.11
0.7	5.84	7.90
0.8	5.97	7.87
0.9	6.08	7.81
1.0	6.11	8.09
Average	5.96	7.83

antigen-binding site. Theoretically, all the antigen-binding sites will face towards the solution-phase. Compared to the sandwich ELISA, PACE had significant improvement of signal strength, hence indicating our rationalization is probably correct.

In the PACE format, the assay for apoA-I can be finished within three hours. Compared to electroimmunoassay, immunoduffusion, or radioimmunoassay, this is a significantly faster method. A sensitivity range of 0.2 to 1.2 μg/ml in our PACE assay is probably adequate for the measurement of serum apoA-I since the average concentration of apoA-I in human serum is 1.2 g/L; many fold higher than the limits of our assay.

In some previous epidemiological studies, apoA-I and B100 showed the most significant association with the severity of angiographically assessed CHD (Aro et al. 1986; Noma et al. 1983). Human HDL particles are made up of two major populations of particles with different composition and metabolism. Both contain apoA-I but only one contains ApoA-II. HDLs that contain only apoA-I (Lipoprotein A-I) can increase cellular cholesterol efflux from cultured cells in vitro. HDLs that contain both apoA-I and apoA-II (Lipoprotein A-I:A-II), however, do not increase cholesterol efflux (Barkia et al. 1991). The ligands that recognize the cell surface HDL-binding sites have been identified as apoA-I, apoA-IV, and apoA-II. It is proposed that apoA-I and apoA-IV play the role of agonists and apoA-II that of antagonist of cholesterol efflux (Barbaras et al. 1990). It is recently shown that lipoprotein A-I:A-II can inhibits the lipoprotein A-I promoted cholesterol efflux of cholesterol-preloaded adipose cells (Barkia et al. 1991). An assay for HDL will be more specific for assessment of CHD risk if lipoprotein A-I:A-II can be removed first by anti-apoA-II antibody.

The variations of apolipoprotein assay are mainly due to individual differences and analytical errors (Evans and Laker 1995). ApoA-I concentrations can increase up to 30% (Rifai et al. 1989) and apoB100 up to 60% (Knopp et al. 1985) during pregnancy. Smokers have significantly lower apoA-I (-4.2%) (Craig et al. 1989). Long-term study in normal subjects has shown that heavy alcohol consumption increases apoA-I concentration (Masarei et al. 1986). It is probably due to the induction of apolipoprotein synthesis in the liver (Luoma et al. 1982). One of the causes of analytical variation in measuring apoA-I level is due to posture. It has been shown that apoA-I level will decrease 12% when the subject changes from standing to lying (Miller et al. 1992). Altered sympathetic nervous system activity may partly explain postural changes (Howes et al. 1987). Different storage methods also contribute to assay variations. The concentration of apoA-I increases with the time of storage at either room temperature or 4°C. These changes in apparent apoA-I concentrations are believed to be from structural changes in HDL leading to altered exposure of the antigenic sites (Wang et al. 1989).

The standard used in this study is pure apoA-I. In order to use the assay for serum samples, it should be tested for nonspecific interferences that may affect the accuracy and precision of the results. Most of the manifestations of interferences can be generally classified as 'matrix effects'. Serum may contains autoantibodies, complement and rheumatoid factor which can lead to a reduction in apparent concentration of the analyte. Moreover, serum may contain enzyme inhibitors, catalysts or cofactors which will interfere the enzyme signal generating system.

Chapter 5

General Conclusions

The contribution of HDL and LDL in the development of atherosclerosis is well understood. Their relationships are true both for morbidity (Gordon et al. 1977) and mortality (Wilson et al. 1988) from CHD. ApoA-I and apoB100 have been suggested to replace HDL- and LDL-cholesterol as better parameters for the assessment of CHD risk (Srinivasan and Berenson 1995; Bhatnagar and Durrington 1991; Laker and Evans 1996). In this study, human serum apoA-I and apoB100 were isolated and their antibodies produced. Using these antibodies as immuno-reagents, we have attempted to develop ELISA for the measurement of these apolipoproteins.

A number of ELISA methods for human apoA-I have been published. In these studies, the antibodies produced have different binding affinities to lipoproteins and free apolipoproteins. It was suggested that sample should be pretreated to expose all the antigenic sites before the estimation of the apolipoprotein level. Incubation at 52°C for 3 hours, denaturation by 6 M guanidine hydrochloride and pretreatment with Tween20 are some of the methods that have been suggested. However, these pre-treatments are time-consuming and made the assay more complicated, as well as subjecting the antibodies to conditions which might affect their binding affinities (Wong et al. 1985). Hence, a monoclonal antibody which can bind to the free and lipoprotein-bound-apolipoprotein with the same affinity will be a more desirable antibody. A monoclonal antibody that can recognize all the possible forms in which the apolipoprotein can exist will enhance its utility for detection of apolipoprotein in the serum environment without pre-treatment.

Another advantage of the ELISA assay developed in this study is the time it takes to perform the assay. Three hours is considered to be one of the most rapid assays for apoA-I. When compared to electroimmunoassay, immunoduffusion, or radioimmunoassay which require several days to be completed, the assay developed in this study is more convenient for the clinical use. In the preliminary optimization of the assay, the antibody/antigen reaction reached equilibrium after incubation at 37°C for one hour. Prolonging the incubation time did not increase the signal strength significantly (results not shown). AB6 probably has a strong affinity to interact with apoA-I.

Finally, the assay for apoA-I described here offer some other advantages over previously described apoA-I immunoassay: no centrifugation steps are required, radioisotopes are not used, and the monoclonal antibody which are highly specific is available in unlimited supply and in large quantity, and are easily purified.

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